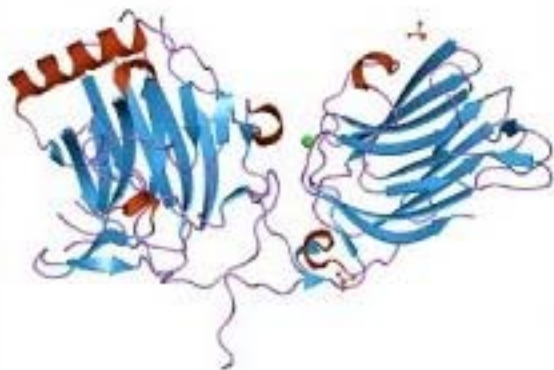


VITAMIN K

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PREFACE

Vitamin K has emerged to prominence in relation to the treatment of deep vein thrombosis that is a major health problem today. Individual variations in the expression of proteins connected with vitamin K activity make for a need to carefully titrate amounts of oral coumadin (warfarin) used to control hypercoagulability. This volume, not only covers the health- and disease-related functions of vitamin K but, importantly, reviews the proteins and genes that are involved in the functions of this vitamin.

To immediately relate individual status with regard to this vitamin, S. L. Booth and A. A. Rajabi initiate the volume with a review: "Determinants of vitamin K status in humans." Several works follow that consider the basic science aspects of the actions of the vitamin, starting with: "VKORC1 and the vitamin K cycle" by A. A. Garcia and P. H. Reitsma. "The vitamin K cycle" is the title of a contribution by J. Oldenburg, M. Marinova, C. Müller-Reible, and M. Watzka. Next, the "Structure, function, and mechanism of cytosolic quinone reductases" is offered by M. A. Bianchet, S. B. Erdemli, and L. M. Amzel, X. Gong, R. Gutala, and A. K. Jaiswal consider: "Quinone oxidoreductases and vitamin K metabolism." "The structure and function of vitamin K epoxide reductase" is the topic reviewed by J.-K. Tie and D. W. Staord. "Vitamin K-dependent carboxylation" is the contribution of K. L. Berkner and "Vitamin K-dependent γ -glutamylcarboxylation: An ancient posttranslational modification" is authored by P. K. Bandyopadhyay, L. Bellido-Martín, and P. García de Frutos write on "Vitamin K-dependent actions of Gas6." "Vitamin K₂-mediated apoptosis in cancer cells: Role of mitochondrial transmembrane potential" is contributed by T. Shibayama-Imazu, T. Aiuchi, and K. Nakaya and, as the last chapter in this section, R. Wallin, N. Wajih, and S. M. Hutson review: "VKORC1: A warfarin-sensitive enzyme in vitamin K metabolism and biosynthesis of vitamin K-dependent blood coagulation factors."

Under the general subject of vitamin K and its relation to disease, V. Siguret, E. Pautas, and I. Gouin-Thibault cover: "Warfarin therapy: Influence of pharmacogenetic and environmental factors on the anticoagulant response to warfarin." G. J. Merli and J. Fink report on: "Vitamin K and thrombosis." A. Girolami, R. Scandellari, M. Scapin, and S. Vettore write on: "Congenital bleeding disorders of the vitamin K-dependent clotting factors." H. Arai, K. Nagai, and T. Doi offer: "Role of growth arrest-specific gene (Gas6) on diabetic nephropathy." "Vitamin K and bone health in adult humans" is the subject of a review by S. Bügel, and C. Heiss.

L. M. Hoesel, U. Wehr, S. Wenisch, I. Drosse, V. Alt, C. Meyer, U. Horas, M. Schieker, and R. Schnettler write on: “Diagnosis of osteoporosis with vitamin K as a new biochemical marker.” Finally, T. Mizuta and I. Ozaki report on: “Hepatocellular carcinoma and vitamin K.”

The structure reproduced on the cover is that of the C-terminal LG domain pair of human Gas6 [protein] from the Protein Data Bank (MSD atlas), ID 1h30, T. Sasaki, *et al.* (2002). Crystal structure of the C-terminal fragment of growth and arrest-specific protein Gas6. Receptor tyrosine kinase activation by laminin G-like domains. *J. Biol. Chem.* **277**, 44164–44170.

The watchful eyes of Renske van Dijk and Tari Broderick at Academic Press/Elsevier continue to shepherd these volumes to completion with the thanks of the Editor-in-Chief.

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DETERMINANTS OF VITAMIN K STATUS IN HUMANS

Sarah L. Booth^{*} and Ala Al Rajabi^{*}

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Abstract

To understand the role of vitamin K in human health, it is important to identify determinants of vitamin K status throughout the life cycle. Our current understanding of vitamin K physiology and metabolism only partially explains why there is wide interindividual variation in vitamin K status, as measured by various biochemical measures. Dietary intake of vitamin K is one of the primary determinants of vitamin K status, and intakes vary widely among age groups

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and population subgroups. How dietary sources of vitamin K are absorbed and transported varies with the form and food source of vitamin K. Likewise, the role of plasma lipids as a determinant of vitamin K status varies with the form of vitamin K ingested. There is also some evidence that other fat-soluble vitamins antagonize vitamin K under certain physiological conditions. Infants are at the greatest risk of vitamin K deficiency because of a poor maternal–fetal transfer across the placenta and low vitamin K concentrations in breast milk. During adulthood, there may be subtle age-related changes in vitamin K status but these are inconsistent and may be primarily related to dietary intake and lifestyle differences among different age groups. However, there is some suggestion that absence of estrogen among postmenopausal women may be a determinant of vitamin K status. Genetics may explain some of the observed interindividual variability in vitamin K, but to date, there are few studies that have systematically explored the associations between individual genetic polymorphisms and biochemical measures of vitamin K status. © 2008 Elsevier Inc.



I. INTRODUCTION

An inadequate diet is an important component in the multifactorial nature of numerous diseases. Infants are born deficient in vitamin K, and, when unsupplemented, are at risk of hemorrhage due to vitamin K deficiency bleeding (VKDB) within the first few months of life. In contrast, frank dietary vitamin K deficiency in adults, without accompanying illness or predisposing factors, is rare. However, poor vitamin K nutrition has been recently linked to several chronic diseases associated with abnormal calcification, including osteoporosis (Kaneki *et al.*, 2006), vascular calcification (Vermeer and Braam, 2001), and osteoarthritis (Neogi *et al.*, 2006). To understand the impact of vitamin K nutrition on health, it is necessary to assess the determinants of vitamin K nutritional status throughout the life cycle.



II. ASSESSMENT OF VITAMIN K STATUS

A. Function

Vitamin K is a cofactor specific to the formation of γ -carboxyglutamyl (Gla) residues from specific glutamate residues in certain proteins. The Gla residues in these vitamin K-dependent proteins confer calcium-binding properties, hence functions, as discussed in greater detail in this volume. Coumarin-based oral anticoagulants, such as warfarin, inhibit coagulation by decreasing the γ -carboxylation of the Glu residues in vitamin K-dependent coagulation proteins. The antagonism of vitamin K results in the secretion of undercarboxylated proteins that have reduced calcium-binding properties.

As demonstrated in controlled metabolic studies in humans, inadequate amounts of available vitamin K in the diet also result in the secretion of undercarboxylated vitamin K-dependent proteins (Booth *et al.*, 2000, 2003; Ferland *et al.*, 1993).

B. Biochemical measures

Although dietary intake has historically been considered the primary determinant of vitamin K status (Booth and Suttie, 1998), other factors are also emerging. Biochemical markers may be more preferable than dietary assessment alone because the former captures the role of both dietary and nondietary determinants of vitamin K status.

1. Circulating concentrations of vitamin K

Circulating phyloquinone concentrations are the primary form in circulation. Plasma phyloquinone concentrations reflect dietary intake over the previous 24 h, and have high intra- and interindividual variation compared to other fat-soluble vitamins (Booth *et al.*, 1997). Less is known about the diet–plasma associations for menaquinones. As discussed in Section III.B, there is controversy regarding the interdependence of circulating forms of vitamin K with plasma lipids.

2. Coagulation times

The prothrombin time (PT), also expressed as an International Normalized Ratio (INR), and activated partial thromboplastin time (APTT) are routine tests of coagulation that can reflect vitamin K deficiency. These tests are nonspecific because prolongation of these measures is also indicative of hepatic dysfunction, hematologic disease unrelated to vitamin K deficiency, and multiple other acute or chronic conditions. PT becomes prolonged only when the prothrombin concentrations drops below 50% of normal, demonstrating its low sensitivity for detecting vitamin K deficiency (Suttie, 1992). In otherwise healthy adults, sustained intakes as low as 10 $\mu\text{g}/\text{day}$ of phyloquinone for several weeks do not prolong PT. In contrast, more sensitive measures do respond to dietary restriction and supplementation and are more appropriate for identifying determinants of vitamin K status.

3. Undercarboxylated vitamin K-dependent proteins

Measurement of circulating undercarboxylated proteins is currently considered to be a more sensitive indicator of vitamin K deficiency. Undercarboxylated prothrombin, also known as PIVKA-II (*p*roteins *i*nduced in the *v*itamin *K* *a*bsence or *a*ntagonism-*f*actor *I*I), has advantages in that it detects abnormalities in prothrombin before the prolongation of PT. Most infants with vitamin K deficiency have elevated PIVKA-II concentrations,

but an elevated PIVKA-II concentration is not necessarily a predictor of hemorrhagic disease. PIVKA-II has also been reported to increase in response to low-dose (1 mg) warfarin (Camilo *et al.*, 1998) and vitamin K dietary restriction (Booth *et al.*, 2001; Ferland *et al.*, 1993). Osteocalcin is one of the most abundant noncollagenous proteins in bone and is thought to act as a regulator of bone mineral maturation, as described in detail elsewhere in this volume. The mineral-binding capacity of osteocalcin is dependent on the vitamin K-dependent γ -carboxylation of its three glutamate residues, such that partially carboxylated osteocalcin may have reduced binding to the mineral in bone. The proportion of serum osteocalcin [expressed as either %ucOC (uncarboxylated osteocalcin) or ucOC/tOC] that is not carboxylated is used as a sensitive marker of vitamin K status. A high proportion of ucOC is indicative of poor vitamin K status. In contrast, the absolute concentrations of ucOC are not particularly meaningful in terms of vitamin K status because these values are dependent on the total amount of osteocalcin available for carboxylation. For example, absolute levels of ucOC may decrease in response to vitamin K supplementation but if the total osteocalcin also decreases because of other factors, then the proportion of osteocalcin that is not carboxylated will remain unchanged, indicative of a null effect of vitamin K. Although there is a weak negative correlation between circulating concentrations of phyloquinone and %ucOC, %ucOC is not strong enough to have predictive value as a measure of individual vitamin K status. Recent studies also suggest that the determinants of %ucOC differ from those of serum phyloquinone (Shea *et al.*, 2007), which emphasize the limitations of using a single biochemical measure as a measure of individual vitamin K status.

4. Urinary measures

Metabolites of vitamin K can now be measured in urine and respond to dietary manipulation of vitamin K (Harrington *et al.*, 2007). It is anticipated that these metabolites will provide a novel marker for assessment of overall vitamin K status. A second urinary measure for which there has been more collective experience is the measurement of urinary Gla excretion. Gla cannot be recycled and is excreted in urine during the turnover of individual vitamin K-dependent proteins. Urinary Gla excretion is an overall measure of vitamin K-dependent proteins, including those for which there are currently no known functions (Ferland, 1998). Urinary Gla excretion is highly correlated with lean body mass so it is usually corrected for urinary creatinine excretion (Sokoll and Sadowski, 1996). In metabolic studies, urinary Gla excretion has been shown to decrease and increase in response to vitamin K dietary restriction and supplementation, respectively (Booth *et al.*, 1999; Ferland *et al.*, 1993).

III. NONGENETIC DETERMINANTS

A. Dietary intakes of vitamin K

1. Dietary forms and sources of vitamin K

The term “vitamin K” represents a family of compounds with a common chemical structure, 2-methyl-1,4-naphthoquinone (Fig. 1.1). *Phylloquinone*, or vitamin K₁, is a compound present in all photosynthetic plants (Gross *et al.*, 2006). In the human diet, phylloquinone is the predominant dietary form of vitamin K and is present in foods of plant origin. In general, green leafy vegetables contain the highest known phylloquinone concentrations and contribute ~60% of total phylloquinone intake (McKeown *et al.*, 2002; Thane *et al.*, 2002). Certain plant oils and margarine, spreads, and salad dressings derived from these plant oils are also important dietary sources of phylloquinone (Peterson *et al.*, 2002; Piironen *et al.*, 1997). *Menaquinones* are primarily of bacterial origin and differ in structure from phylloquinone in their 3-substituted lipophilic side chain. The major menaquinones contain 4–10 repeating isoprenoid units, indicated by MK-4 to MK-10; forms up to 13 isoprenoid groups have been identified. *Menaquinone-4* (MK-4) is not a major constituent of bacterial production; instead it is alkylated from menadione present in animal feeds or is the product of tissue-specific conversion directly from dietary phylloquinone (Davidson *et al.*, 1998; Thijssen *et al.*, 2006). Because poultry feed is a rich source of menadione, which is subsequently converted to MK-4 in certain tissues, poultry products are the primary dietary sources of MK-4 in the US food supply (Elder *et al.*, 2006; Ferreira *et al.*, 2006). *Menaquinone-7* (MK-7) is primarily found in natto, a soybean product that is fermented using *Bacillus natto*. Although certain cheeses contain some menaquinones (Schurgers and Vermeer, 2000), generally the longer chain menaquinones are not thought to be present in the food supply. Very little is known about the contribution of dietary menaquinones to overall vitamin K nutrition, and although it is a generally held belief that ~50% of the daily requirement for vitamin K is supplied by the gut flora through the production of endogenous menaquinones, there is insufficient experimental evidence to support this conviction (Suttie, 1995). In one small study in individuals with acute bacterial

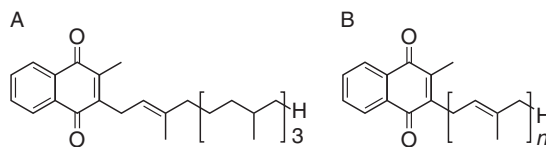


Figure 1.1 Dietary forms of vitamin K: phylloquinone (A) and menaquinones (B).

overgrowth as induced by omeprazole, menaquinones produced by these bacteria had some contribution to vitamin K nutriture during dietary phyloquinone restriction, but not enough to restore normal vitamin K status (Paiva *et al.*, 1998).

2. Dietary intakes

An adequate intake (AI) for vitamin K was set at 120 and 90 $\mu\text{g}/\text{day}$ for men and women, respectively (Table 1.1 IOM, 2001). The AI is determined by the reported median intake among adults in the United States (IOM, 2001). In infants, the AI is set at 2–2.5 $\mu\text{g}/\text{day}$ based on the estimated phyloquinone intake from breast milk. Infants exclusively breast-fed have an estimated daily intake of 0.5–2.6 μg (IOM, 2001), and they are at greater risk for vitamin K deficiency because breast milk is a poor dietary source of vitamin K. In contrast, infant formulas contain appreciable amounts of phyloquinone so the average daily intake of US infants 2–6 months of age is 63 μg (IOM, 2001), which is far in excess of the AI for this age group. The AI for children aged 1 year and older is set on the basis of the median intake for each age group as estimated in US national surveys (Table 1.1). The adequacy of these intakes for health has not yet been determined because there are currently no physiological outcomes available that can be

Table 1.1 Adequate intakes for vitamin K (IOM, 2001)

Age group	Adequate intake ($\mu\text{g}/\text{day}$)
Infants	
0–6 months	2
7–12 months	2.5
Children	
1–3 years	30
4–8 years	55
9–13 years	60
14–18 years	75
Men	
19–70 years	120
>70 years	120
Women	
19–70 years	90
>70 years	90
Pregnant and lactating women	
14–18 years	75
19–50 years	90

reliably used to assess adequacy. Recent surveys in North America, Europe, and Asia indicate that wide ranges in dietary intakes of phyloquinone exist across different geographic regions and among different ages. Subgroups of populations with an average reported phyloquinone intake below current recommendations include nursing home residents in Hong Kong (Tse *et al.*, 2002), and adults (Duggan *et al.*, 2004; Thane *et al.*, 2002; Yan *et al.*, 2004) and children (Prynne *et al.*, 2005) in the United Kingdom. Furthermore, phyloquinone intakes have been decreasing over the last two decades in the United Kingdom, consistent with a concomitant decline in leafy green vegetable consumption (Thane *et al.*, 2006a). In contrast, reported average phyloquinone intakes among adults in Germany (Mensink and Beitz, 2004), the Netherlands (Schurgers *et al.*, 1999), Japan (Kimura *et al.*, 2003), and Northern China (Yan *et al.*, 2004) are within the current dietary recommendations. Variation in the type of dietary assessment used and the source of food composition data may result in measurement errors that contribute to the wide reported ranges of vitamin K intakes within and between subgroups (Booth and Suttie, 1998).

3. Associations with biochemical measures

Dietary restriction of phyloquinone to 35 $\mu\text{g}/\text{day}$ causes rapid decreases in plasma phyloquinone (Table 1.2) and urinary excretion of Gla residues, and increases in the under- γ -carboxylated forms of the vitamin K-dependent proteins, osteocalcin (%ucOC) and prothrombin (PIVKA-II), without affecting classic measures of blood coagulation (Booth *et al.*, 2000, 2003; Ferland *et al.*, 1993; Suttie *et al.*, 1988). The rapidity of the observed changes in these studies suggests that the complete carboxylation of the Gla residues in vitamin K-dependent proteins may require AIs of vitamin K on a daily basis. In reviewing the data, it is of importance to note that most metabolic studies have examined the response of biochemical markers of vitamin K status to phyloquinone supplementation in adults who are already vitamin K-replete. However, recent population-based data suggest that ~15% of adults have plasma phyloquinone concentrations equivalent to those attained with vitamin K dietary restriction (i.e., below 0.5 nmol/liter) (Booth *et al.*, 2004a; Thane *et al.*, 2002). In observational studies, dietary phyloquinone intakes are positively, albeit weakly associated with circulating concentrations of phyloquinone (Thane *et al.*, 2006a) and inversely associated with %ucOC (McKeown *et al.*, 2002). There appears to be an intake threshold of ~200 $\mu\text{g}/\text{day}$ above which there is no longer a linear association with circulating concentrations of phyloquinone when dietary intakes are assessed by food frequency questionnaires (McKeown *et al.*, 2002). This observation most likely reflects limitations in the food frequency questionnaire in assessing high intakes of vitamin K because this threshold effect is not supported by the dietary supplementation studies in which plasma phyloquinone concentrations continue to increase with supplementation of up to 1000 $\mu\text{g}/\text{day}$

Table 1.2 Response of biomarkers of vitamin K status to phyloquinone restriction and supplementation

References	Participants	Intervention with phyloquinone	Plasma phyloquinone	Serum %ucOC
(Knapen <i>et al.</i> , 1989)	100 women 55–75 years 25–40 years	14-day @ 1 mg/day	NA	↓ (55–75 years) No Δ (24–40 years)
(Knapen <i>et al.</i> , 1993)	145 women 20–85 years	14-day @ 1 mg/day	NA	↓
(Douglas <i>et al.</i> , 1995)	10 women 52–73 years	14-day @ 1 mg/day	NA	↓
(Craciun <i>et al.</i> , 1998)	8 women mean age: 35 years	30-day @ 10 mg/day	NA	↓
(Booth <i>et al.</i> , 1999)	36 men and women 20–40 years 60–80 years	5-day @ 400 μg/day	↑	↓
(Binkley <i>et al.</i> , 2000)	112 men and women 18–30 years ≥65 years	14-day @ 1 mg/day	↑	↓
(Booth <i>et al.</i> , 2001)	15 men and women 20–40 years	15-day @ 10 μg/day	↓	↑
(Binkley <i>et al.</i> , 2002)	10 men and women 19–36 years	10-day @ 200 μg/day	↑	↓
		7-day @ 0.5–2 mg/day	NA	↓
	100 men and women mean age: 26 years	14-day @ 250 μg/day	↓	↑
(Booth <i>et al.</i> , 2003)	21 women 60–80 years	14-day @ 375–1000 μg/day	↑	↓
		30-day @ 18 μg/day	↓	↑
		30-day @ 86–200 μg/day	No Δ	No Δ
		15-day @ 450 μg/day	↑	No Δ

(Binkley *et al.*, 2002). More recently, MK-4 and MK-7 plasma or serum concentrations have been measured in certain populations that are supplemented and/or consume high intakes of fermented soybean products, respectively (Tsugawa *et al.*, 2006). Neither PIVKA-II nor urinary Gla excretion measurements have been used to assess associations between vitamin K status and dietary intakes in observational studies.

B. Absorption, transport, and metabolism of vitamin K

Most disease states that detrimentally influence vitamin K status are related to malabsorption or other gastrointestinal disorders in origin, including biliary atresia, cystic fibrosis, celiac disease, and short bowel syndrome, as extensively reviewed elsewhere (Savage and Lindenbaum, 1983). The absorption of vitamin K has not been well studied in healthy adults, particularly in context of dietary sources and different vitamin K forms and in regards to different stages of the life cycle. In common with other fat-soluble vitamins, phyloquinone is absorbed from the proximal intestine and the absorption is dependent on bile and pancreatic secretion (Shearer *et al.*, 1974). In the intestine, phyloquinone is incorporated into nascent chylomicron particles that are secreted directly into the lymph and ultimately into peripheral circulation. Phyloquinone remains associated with these particles during delipidation in circulation and subsequent uptake by the liver. Vitamin K is not known to have a carrier protein; instead, triglyceride-rich lipoproteins (TRL), primarily chylomicron remnants and very low density lipoproteins (VLDL), are thought to be the main transporters of phyloquinone (Kohlmeier *et al.*, 1996; Lamon-Fava *et al.*, 1998; Schurgers and Vermeer, 2002). Plasma phyloquinone concentrations are consistently correlated with triglycerides (Tsugawa *et al.*, 2006). Age-related differences in the clearance of phyloquinone from circulation may in part explain the documented high interindividual differences in plasma phyloquinone concentrations (Booth *et al.*, 1997; Thane *et al.*, 2006b). There is current controversy regarding the adjustment of plasma phyloquinone for total plasma lipids to assess the predictive value of fasting plasma phyloquinone concentrations on health outcomes, such as hip fracture risk (Cham *et al.*, 1999; Traber and Jialal, 2000). Absolute plasma phyloquinone concentrations are higher among individuals with elevated triglyceride concentrations. Fasting plasma phyloquinone and triglyceride concentrations are both higher in older adults compared to younger adults (Booth *et al.*, 2002; Sadowski *et al.*, 1989), but when phyloquinone concentrations are adjusted for triglycerides, the adjusted plasma phyloquinone concentrations are lower in the older adults compared to the younger adults (Sadowski *et al.*, 1989). If adjusted fasting plasma phyloquinone concentrations are a reliable measure of body stores, then these results suggest that older adults have

decreased body stores of phyloquinone. However, this has not been consistently substantiated by metabolic data (Table 1.2). In observational studies, it is difficult to interpret the interrelationship between markers of vitamin K and lipid status, and plasma phyloquinone concentrations also have a high intraindividual variability that is thought to be dietary in origin. Less is known about the absorption of menaquinones, but of the limited data available, MK-4 and MK-7 do not appear to be correlated with plasma lipids (Tsugawa *et al.*, 2006).

Phylloquinone is tightly bound to the membranes of plant chloroplasts, and it is less bioavailable compared to phyloquinone obtained from plant oils and/or dietary supplements (Booth and Suttie, 1998). MK-7, administered in the form of natto in equimolar amounts, compared to phyloquinone, administered in the form of spinach, has a peak height difference of more than 10-fold and a half-life of 56 h compared to 7.5 h for phyloquinone (Schurgers and Vermeer, 2000). Whereas all forms of vitamin K appear to be initially associated with TRL, the longer chain menaquinones, including MK-7 and MK-9, are also associated with low-density lipoprotein (LDL). MK-4 has been reported in TRL, LDL, and high-density lipoproteins (HDL). These preliminary data suggest that menaquinones have different transport pathways and distribution, which has implications for transport to extrahepatic tissue, such as bone. Animal data support this differential transport of the different forms of vitamin K because MK-7 shortened the prolonged PT in vitamin K-deficient rats for a longer period compared to phyloquinone or MK-4 (Sato *et al.*, 2002). There are no data on potential interactions between different forms of vitamin K when coadministered.

C. Age

Infants are born deficient in vitamin K because of poor maternal–fetal transfer across the placenta (AAP, 2003). Breast milk contains very low phyloquinone concentrations, which does not appear to be related to the maternal diet, and initially there is limited bacterial colonization of the gut, which could otherwise produce endogenous menaquinones. In addition, coagulation factor concentrations are low at the time of birth, which increases the risk of hemorrhagic bleeding known as VKDB. Vitamin K prophylaxis in the form of a single, intramuscular dose of 0.5–1 mg of phyloquinone is routinely administered as an effective intervention against VKDB. There are few studies on the safety and bioavailability of oral formulations and optimal dosing regimens of vitamin K to prevent late VKDB. Recent data suggest that intramuscular prophylaxis of 0.2 mg of phyloquinone maintains adequate vitamin K status in preterm infants, a group that is at higher risk for VKDB, without evidence of the hepatic overload seen in premature infants receiving the higher doses that are

consistent with current public policy (Clarke *et al.*, 2006). The incidence of VKDB declines at 12 weeks of age, and spontaneous bleeding beyond that age is rare and limited to lipid malabsorption syndromes, as discussed in Section II.B.

As also discussed in Section III.B, there is controversy regarding age-related differences in the vitamin K status among adults, particularly in observational studies. These differences may in part be explained by age-related differences in lipid concentrations. In controlled dietary studies, older adults have been reported to be relatively more resistant to the development of subclinical vitamin K deficiency when fed a low-vitamin K diet (Ferland *et al.*, 1993). It has been proposed that there are age-related differences in absorption and catabolism of phyloquinone, the turnover of vitamin K-dependent proteins is lower in older adults, and/or they have lower vitamin K requirements (Ferland *et al.*, 1993). Alternatively, these data may simply reflect the age-related differences in phyloquinone intakes, hence hepatic stores, prior to participation in metabolic studies. At least one metabolic study demonstrated that subclinical vitamin K deficiency can be created in elderly women following at least 6 weeks of restricted vitamin K intake (Booth *et al.*, 2003). There was one report of an age-dependent effect, with no change in %ucOC in response to vitamin K supplementation among younger women (Knapen *et al.*, 1989), but this has not been a consistent finding across studies (Table 1.2). In a cross-sectional study of healthy Japanese women who resided in a region with high *natto* consumption, women in the ≥ 70 years age group had overall poorer vitamin K status as measured by %ucOC when compared to younger women (Tsugawa *et al.*, 2006). In contrast, the older women had similar circulating levels of both phyloquinone and MK-7 when compared to the younger women (30–49 years age group), which suggests that even though the younger women had the lowest levels of phyloquinone or MK-7 in circulation, there was more vitamin K available for carboxylation of the osteocalcin in bone compared to the other age groups. Unfortunately no dietary data were reported to confirm observations in other studies that older women consume higher intakes of vitamin K, but clearly there are factors other than diet that are contributing to this disparity in vitamin K status among different age groups. Further, these data confirm previous reports on differences in vitamin K status among women of different estrogen status from the Framingham Offspring Study (Booth *et al.*, 2004a), and suggest that estrogen status may be an important determinant of vitamin K status, independent of diet.

D. Role of estrogen

In general, there have been no systematically reported differences in determinants of vitamin K status when compared between men and women. However, estrogen withdrawal at menopause may result in impairment in

vitamin K metabolism (Lukacs *et al.*, 2006). Among early postmenopausal women not using hormonal replacement therapy, there was a higher phyloquinone level (i.e., indicative of superior vitamin K status) and high %ucOC (i.e., indicative of inferior vitamin K status) compared to premenopausal women. Triglycerides were equal across the groups studied; hence the higher phyloquinone levels in the postmenopausal women were not being influenced by elevated triglyceride levels (Lukacs *et al.*, 2006). This suggests that the degree of carboxylation of the osteocalcin due to the presence of vitamin K is not linearly related to the amount of phyloquinone transported. Further, this relationship between the amount of phyloquinone in circulation and the amount of phyloquinone available in osteoblasts to carboxylate osteocalcin may be modulated by the presence of estrogen. Along the same lines of investigation, the change in ucOC was examined in response to two different regimens of hormonal replacement therapy in postmenopausal women (Yasui *et al.*, 2006). In this study, there was a decrease in ucOC which the authors attributed to the effect of hormonal replacement therapy on increased triglyceride levels, which in turn elevated levels of vitamin K available to the bone for carboxylation. However, the proportion of ucOC to total osteocalcin (i.e., a higher %ucOC) actually increased, suggestive of a decline in vitamin K status in the bone in response to hormone replacement therapy. Unfortunately, no measures of circulating concentrations of vitamin K were reported. Collectively, these studies implicate the absence of estrogen as a determinant of vitamin K status in postmenopausal women, although the mechanisms are currently unknown, which limits our ability to interpret the current data.

E. Interactions with other nutrients

A relatively understudied area of vitamin K nutrition is how interaction with other nutrients, in particular the other fat-soluble vitamins, influences vitamin K status. In a recent study by Thane *et al.* (2006a), 10% of the variation in plasma phyloquinone concentrations was explained by intake of other fat-soluble vitamins, including vitamins E and A. In animal studies, relatively small increments in supplemental α -tocopherol appear to affect the tissue content of vitamin K, particularly under conditions of vitamin K deficiency or antagonism (Abdo *et al.*, 1986; Frank *et al.*, 1997; Tovar *et al.*, 2005). Doses of vitamin E at the tolerable upper limit of 1000 IU RRR- α -tocopherol can result in an increase in PIVKA-II in adults with normal coagulation status (Booth *et al.*, 2004b). Reports of bleeding episodes have been limited to individuals taking vitamin E supplements concurrent with vitamin K antagonists, such as warfarin (Corrigan and Marcus, 1974). One proposed mechanism for the interactions between vitamins E and K is the competitive inhibition of vitamin K-dependent carboxylase by the vitamin E metabolite, tocopherol quinone (Dowd and Zheng, 1995). An alternative

hypothesis is that supraphysiologic doses of vitamin E interfere with vitamin K activation of the pregnane X receptor (PXR) (Landes *et al.*, 2003). In one small rodent study, intake of a lutein mixture preserved with vitamin E resulted in decreased absorption, uptake, and transport of phyloquinone and MK-4 (Mitchell *et al.*, 2001). Similarly, a hemorrhagic toxicity was induced in rats following treatment with pharmacological doses of vitamin E and β -carotene (Takahashi, 1995), and various retinoids (McCarthy *et al.*, 1989). The mechanisms by which vitamin A may directly antagonize vitamin K have yet to be elucidated, but warrant further exploration. In a study by Szulc *et al.* (1993), there was an inverse association between ucOC and serum 25(OH)D concentrations, and a subsequent reduction in ucOC by vitamin D supplementation. These findings cannot be explained by our current understanding of the biochemical role of vitamin K, but suggest that vitamin D may influence the ucOC level. In contrast, serum 25(OH)D and phyloquinone concentrations are not correlated (Shea *et al.*, 2007; Thane *et al.*, 2006b).

F. Antibiotics and other medications

Vitamin K deficiency is often attributed to broad-spectrum antibiotic use, presumably by a reduction in endogenous menaquinone production by intestinal bacteria (Lipsky, 1994). The use of certain antibiotics containing an *N*-methylthiotetrazole side chain (e.g., cefamandole, cefoperazone) can result in a vitamin K antagonism, possibly through a weak inhibition of the γ -carboxylase among individuals with poor vitamin K status. However, any direct effect of broad-spectrum antibiotic use on menaquinone production, hence vitamin K status, among individuals with an adequate dietary intake remains controversial (Conly and Stein, 1994; Lipsky, 1994). Salicylates and anticonvulsants are also associated with vitamin K deficiency in some individuals. Those who take these medications and have coexisting compromised nutritional status may be at particular risk for vitamin K deficiency.

G. Lifestyle factors

By nature of their study design, observational studies usually preclude the ability to isolate the effects of a single nutrient from those of the dietary patterns associated with high intakes of food(s) rich in that nutrient. A high phyloquinone intake may simply be a marker for an overall healthy diet that includes high vegetable consumption given that green leafy vegetables are consistently the primary form of vitamin K in the diet (Erkkila *et al.*, 2005). Further, individuals with high phyloquinone intake tend to consume more fruits and vegetables, more dietary fiber, and less saturated fat, in addition to taking more dietary supplements (Braam *et al.*, 2004). The consistent

inverse associations between smoking and plasma phyloquinone concentrations (Braam *et al.*, 2004; Thane *et al.*, 2006b) may also be explained by this association between phyloquinone intake and healthy lifestyle. Likewise, soybean is a rich dietary source of MK-7 such that high plasma MK-7 concentrations may be indicative of an overall healthy diet.



IV. GENETIC DETERMINANTS

A. Interindividual variability

There has been a recent interest in the role of genetics as a determinant of the interindividual variation in nutrient status. Similar to other nutrients, the response to dietary vitamin K supplementation under controlled conditions has been shown to widely vary among healthy individuals (Erkkila *et al.*, 2004). Nongenetic determinants, as reviewed in Section III, account for ~20% of the interindividual variation in vitamin K status when studied in primarily Caucasian adults (Shea *et al.*, 2007; Thane *et al.*, 2006a). In one study of 264 sib-pairs of Caucasian origin, the biochemical measures for vitamin K status were not significantly heritable, hence did not explain much of the observed interindividual variability (Shea *et al.*, 2007). What is not known though is the extent to which genetics explains interindividual variability in vitamin K status when comparing populations of diverse ethnic and racial origin. Potential genetic determinants of vitamin K status include variation in the genes involved in the transport or uptake of vitamin K into the tissues, and the genes involved in the tissue-specific availability and recycling of vitamin K. At the time of this writing, there have been very few published studies that had assessed the effect of these polymorphisms on measures of vitamin K status.

B. Apolipoprotein E

The gene encoding apolipoprotein E (ApoE) codes for a 34-kDa lipoprotein primarily found on the TRL (VLDL and chylomicrons) and HDL (Das *et al.*, 1985). ApoE is a ligand for the LDL receptor and other TRL receptors, and is therefore largely responsible for the cellular uptake of TRL. Three common alleles, made up of amino acid substitutions at positions 112 and 158, are found in the population and are referred to as E2, E3, and E4 (Eichner *et al.*, 2002). The various ApoE isoforms interact differently with the LDL and other lipoprotein receptors, ultimately altering circulating levels of cholesterol and triglycerides (Eichner *et al.*, 2002). The ability of ApoE to clear vitamin K-rich intestinal lipoproteins from circulation is greatest with E4 and least with E2 (Kohlmeier *et al.*, 1995; Weintraub *et al.*, 1987). In a study of hemodialysis patients, serum phyloquinone

concentrations in patients with the E3/4 or E4/4 genotypes were less than half that of patients with the genotype E3/3. Those with the genotype E2/3 or E2/2 had the highest serum phyloquinone concentrations (Kohlmeier *et al.*, 1995). It is thought that the liver is the primary organ for vitamin K uptake and carriers of the E4 allele may have increased hepatic vitamin K uptake and decreased vitamin K in circulation, hence decreased levels of vitamin K available for γ -carboxylation of osteocalcin in the bone (Newman *et al.*, 2002). This is supported by the observation that E4 homozygotes require, on average, a higher dose of warfarin, a vitamin K antagonist, compared to non-E4 carriers (Kohnke *et al.*, 2005). A higher warfarin dose in E4 homozygotes would be necessary to compensate for the increased hepatic vitamin K uptake. In contrast, in a study of healthy older adults from China and the United Kingdom, carriers of the E4 allele had higher plasma phyloquinone concentrations and lower %ucOC (Yan *et al.*, 2005). The authors of this study suggested that the carriers of the E4 allele had slower clearance of the TRL remnants from circulation, and subsequently more phyloquinone available for uptake in the bone. In another study, ethnic differences in ApoE genotype, with Gambian postmenopausal women having the highest frequency of E4 allele (32.6%) compared to UK and Chinese postmenopausal women (13.8% and 6%, respectively), were associated with differences in %ucOC, but not plasma phyloquinone concentrations (Beavan *et al.*, 2005). Clearly, more research is required to determine the direction and magnitude of this putative effect that ApoE genotype has on vitamin K nutrition status.

C. Vitamin K epoxide reductase

Vitamin K epoxide reductase (VKOR) is a necessary enzyme for the vitamin K cycle, as discussed elsewhere in this volume. Common polymorphisms and haplotypes within the *VKORC1* gene have been associated with interindividual variability in warfarin dose (Li *et al.*, 2006; Montes *et al.*, 2006; Obayashi *et al.*, 2006). Because warfarin is a vitamin K antagonist that works by directly inhibiting VKOR and thus the recycling of vitamin K, polymorphisms affecting vitamin K recycling in the liver may modulate vitamin K status. One recent study has shown that single nucleotide polymorphisms and haplotypes within the *VKORC1* locus were associated with ucOC and PIVKA-II concentrations in a Chinese cohort (Wang *et al.*, 2006). Paradoxically, the genotype that was associated with lower PIVKA-II concentrations, hence better vitamin K status, was also associated with greater risk of vascular disease. The ucOC was expressed as an absolute concentration and as discussed in Section II.B.3, it is the percentage of the osteocalcin that is carboxylated that provides an insight into the availability of vitamin K. Unfortunately there were also no corresponding data on

plasma phylloquinone concentrations in this cohort to confirm vitamin K status relative to the individual genotypes.

D. Gamma glutamyl carboxylase

Gamma glutamyl carboxylase (GGCX) is necessary for the carboxylation of vitamin K-dependent proteins, as discussed in greater detail in this volume. Common variations within the GGCX locus have been analyzed for association with transcriptional activity and required warfarin dose ([Vecsler et al., 2006](#)). However, little has been done to examine the effect of these polymorphisms on direct measures of vitamin K status or γ -carboxylation of extrahepatic proteins such as osteocalcin.

V. CONCLUSIONS AND FUTURE DIRECTIONS

To understand the role of vitamin K in human health, it is necessary to identify determinants of vitamin K status throughout the life cycle. To date, the research in this area has been focused on the roles of diet, age, and, to a lesser extent, circulating lipid concentrations in explaining the interindividual variation in vitamin K status. Dietary intakes vary widely in the amount and forms consumed among age groups and different subgroups of populations. This variation in turn influences how vitamin K is absorbed and transported. Likewise, the role of plasma lipids as a determinant of vitamin K status varies with the form of vitamin K ingested. Our current understanding of vitamin K physiology and metabolism only partially explains the sources of interindividual variation in vitamin K status, as measured by various biochemical measures. It is plausible that dietary intakes of vitamin K contribute more than can be currently quantified, but methodological limitations may attenuate diet–plasma correlations. Despite its role as a primary determinant of vitamin K status, there are insufficient dose–response data and a poor understanding of the physiological relevance of the biological markers from which to establish recommended dietary allowances ([Sutor and Meyers, 2007](#)).

It is well recognized that infants are the only age group at risk of frank vitamin K deficiency as characterized by VKDB. There are few published studies in children and adolescents from which to evaluate determinants of vitamin K status that may be potentially unique to these age groups. In contrast, most of our understanding about vitamin K has been focused on the aging process. During adulthood, there may be subtle age-related changes in vitamin K status but the literature is inconsistent. There is some suggestion that absence of estrogen among postmenopausal women may be a determinant of vitamin K status.

Genetics may explain some of the observed interindividual variability in vitamin K but to date, there are few studies that have systematically explored the associations between individual genetic polymorphisms and biochemical measures of vitamin K status. This appears to be a promising area of research that may also serve to explain some of the inconsistent data on the putative roles of age and plasma lipid concentrations as determinants of vitamin K status.

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VKORC1 AND THE VITAMIN K CYCLE

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Abstract

Vitamin K epoxide, a product of γ -carboxylation, must be rapidly recycled to its reduced form before it can be reused. The set of sequential reactions that guarantees vitamin K recycling is known as the vitamin K cycle. This review is focused on biochemical and genetic aspects of a recently characterized key enzyme of the cycle named vitamin K epoxide reductase complex subunit 1 (*VKORC1*). This 163-amino acid long protein (18 kDa) is responsible for vitamin K reduction. Recent studies also demonstrated the existence of a VKOR complex in which protein disulfide isomerase (PDI) and *VKORC1* appear to be tightly associated. PDI is a thioredoxin-like oxidoreductase and chaperone that is present at high concentrations in the endoplasmic reticulum (ER) and that provides electrons for reduction of the CXXC center in *VKORC1*. Genetic studies have shown that the *VKORC1* gene extends over 5126 base pairs on human chromosome 16 and comprises three exons. Mutations in the *VKORC1* gene causes generalized defective vitamin K-dependent clotting factors (VKCFD2) and warfarin resistance (WR). More detailed pharmacogenetic studies have demonstrated a strong association between single nucleotide polymorphisms (SNPs) in the *VKORC1* gene and the requirement of warfarin dosage. Screening for *VKORC1* polymorphisms that affect warfarin dosage could be helpful to tailor dosage at the onset of oral anticoagulant treatment in order to lower thrombosis and bleeding risks. © 2008 Elsevier Inc.

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I. VITAMIN K CYCLE: LIMITING STEP OF CARBOXYLATION

Vitamin K-dependent proteins require carboxylation of key glutamic acid residues for exerting their activity. The best known vitamin K-dependent proteins include the coagulation factors II (prothrombin), VII, IX, X, protein Z, protein S, and protein C. Each of these proteins is γ -carboxylated at several amino terminal glutamyl residues in a vitamin K-dependent process (Furie and Furie, 1988). Other γ -carboxylated proteins not involved in blood clotting have been described as essential elements of diverse physiological processes. Osteocalcin and matrix Gla-protein both act in bone metabolism, whereas growth arrest protein Gas6 plays a role in cell growth and survival. In apoptosis and cell survival, various carboxylated receptor tyrosine kinase ligand proteins (that are crucial members of the gap junction family of proteins) are also involved in intercellular signaling and homeostasis. Finally, four putative carboxylated transmembrane proteins (abbreviated as PRGP1, PRGP2, TmG3, and TmG4) are known, the biological function of which remains to be identified (Kulman *et al.*, 2001; Oldenburg *et al.*, 2006).

The enzyme that usually catalyzes the carboxylation reaction is a vitamin K-dependent γ -carboxylase, which uses oxygen and a reduced form of vitamin K (reduced vitamin K hydroquinone—KH₂) to add a carbon dioxide moiety to glutamic acid, thereby producing γ -carboxyglutamic acid. For the blood coagulation factors, γ -carboxylation by γ -glutamyl carboxylase (GGCX) enables the sequestration of calcium ions, and consequently, the association with negatively charged phospholipid membrane surfaces.

According to *in vitro* studies on the biochemical mechanism of γ -carboxylation, the first step of the reaction consists of tight binding between the γ -carboxylase and the propeptide sequence of the substrate. All the carboxylation reactions for a given substrate occur in a single binding event, that is, while bound, all Glu residues are modified at the same time (Morris *et al.*, 1995). The carboxylation process involves the release of hydrogen from glutamyl residues (Glu), with the generation of a carbanion, that is able to incorporate CO₂, resulting in γ -carboxylated Glu (Gla). In this reaction, vitamin K is oxidized simultaneously to vitamin K 2,3-epoxide (vit. K > O) (Furie *et al.*, 1999). For each glutamate carboxylated, a molecule of vitamin K epoxide (vit. K > O) is formed.

The extent of carboxylation is determined by the relative rate of carboxylation and the dissociation rate of the substrate from the γ -carboxylase. The rate of carboxylation is also dependent upon the level of vitamin K available, while the rate of dissociation between substrate and γ -carboxylase is dependent upon both the propeptide and the number of Gla residues of the substrate (Tie *et al.*, 2000).

The importance of the availability of sufficient vitamin K for the rate of carboxylation also became evident when it was shown that osteocalcin circulating in the blood of normal volunteers only reached full carboxylation when vitamin K supplements were given (Binkley *et al.*, 2002). The propeptide of osteocalcin has the lowest affinity of any of the known vitamin K-dependent proteins, and is therefore a sensitive indicator of vitamin K status from the perspective of carboxylation. Although the Gla domain of osteocalcin has a relatively high affinity for GGCX, it can be assumed that the low affinity of the propeptide combined with less than optimal vitamin K concentration allows the osteocalcin to dissociate before carboxylation is complete and that increased vitamin K levels stimulated the rate of carboxylation (Binkley *et al.*, 2002).

Due to the overall scarcity of vitamin K in cells, vitamin K epoxide (vit. K > O), that is generated as a product of γ -carboxylation, must be rapidly recycled to reduced form, KH₂, in order to sustain further γ -carboxylation reactions. The set of sequential reactions that guarantees vitamin K recycling is known as vitamin K cycle (Fig. 2.1) (Buitenhuis *et al.*, 1990).

The enzyme responsible for the regeneration of vitamin K from vitamin K epoxide (vit. K > O) is *Vitamin K epoxide reductase* (VKOR). This activity was first reported in 1970 (Bell and Matschiner, 1970) but it proved resistant to purification for more than 30 years. It was also noted that administration of warfarin, an anticoagulant drug developed around 1943, caused an increase in the level of vitamin K epoxide (vit. K > O) by blocking enzymatic VKOR activity in the liver. NADPH-dependent vitamin K reductase, formerly known as DT-diaphorase, can also reduce vitamin K quinone to the fully reduced KH₂, but is not capable of reducing vit. K > O. Thus, VKOR is the only enzyme that has been shown to catalyze KO de-epoxidation.

A. *VKORC1* gene

In 2004, the elusive gene encoding VKOR was independently identified by two laboratories (Li *et al.*, 2004; Rost *et al.*, 2004a). Both results were published in the same issue of *Nature*.

Rost *et al.* have studied patients and rats with inherited defects in VKOR activity. They found that mutations in one particular gene occurred in two families with defective vitamin K-dependent clotting factors (VKCFD2), in four families with hereditary warfarin resistance (WR), and in several rat strains with resistance to warfarin-like poisons. They also showed that this candidate *VKOR* gene encodes a small transmembrane protein that is found in the endoplasmic reticulum (ER). They named this protein, *vitamin K epoxide reductase complex subunit 1* (VKORC1), thus taking into consideration the possibility that it might be only one component of a larger complex. The protein produced from the normal gene shows VKOR

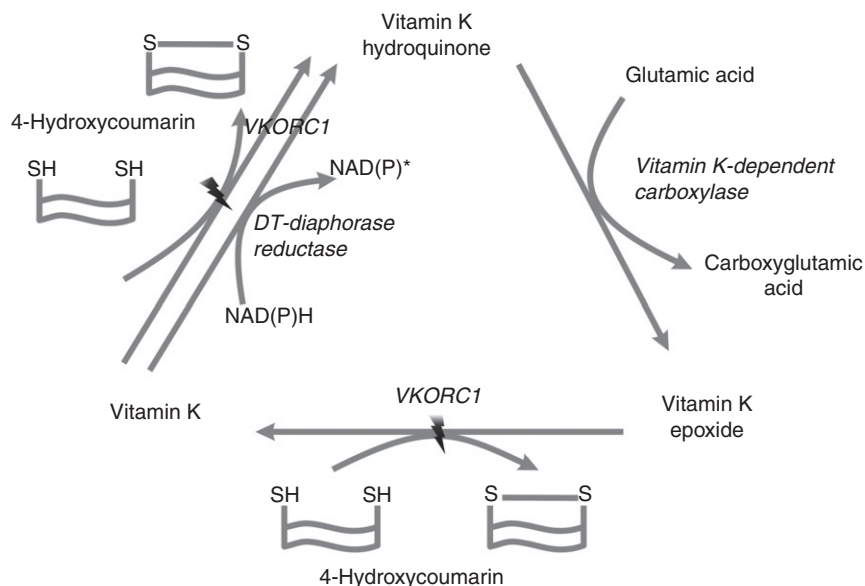


Figure 2.1 Overview of the cycling of vitamin K. The glutamic acids present in several vitamin K-dependent proteins are converted to carboxyglutamic acid by a vitamin K-dependent γ -carboxylase which uses reduced vitamin K hydroquinone (KH_2), carbon dioxide, and oxygen as reactants. Vitamin K epoxide (vit. $K > O$) is produced and must be rapidly recycled to KH_2 in order to be reused in further γ -carboxylation reactions. The reduction of vit. $K > O$ is catalyzed by vitamin K epoxide reductase complex subunit 1 (VKORC1) in two steps. The DT diaphorase, a NADPH-dependent vitamin K reductase, can also reduce vitamin K, but only starting at the vitamin K quinone form. Coumarin-derived anticoagulants such as warfarin and phenprocoumon can inhibit VKORC1 (represented by black lightning), blocking vitamin K recycling.

activity that is inhibited by warfarin. So, by using a positional cloning approach that integrated mapping information from three species, a novel gene that is mutated in VKCFD2 and WR was identified successfully.

Meanwhile, Li *et al.* identified the *VKORC1* gene using another approach (Li *et al.*, 2004). Based on previous papers, they concentrated on a candidate locus on chromosome 16 (Fregin *et al.*, 2002; Kohn and Pelz, 2000). First, genes were ruled out that encode proteins with known or predicted functions. Of the remaining genes encoded in the candidate locus, 13 were selected on the basis of the fact that protein modeling predicted a transmembrane region. This choice was made on the basis of previous findings that VKOR was probably a membrane-bound protein. Then, knockdown experiments were performed using double-stranded small interfering RNA molecules (siRNAs) for each of the 13 gene candidates in A549 cultured cells with high intrinsic VKOR activity. Knockdown of only 1 of the 13 genes resulted in significant reduction of VKOR activity.

The putative *VKOR* gene was cloned and overexpressed in insect cells to confirm VKOR function and warfarin sensitivity. Apparently, this was the first instance that a mammalian gene was identified using the siRNA gene knockdown approach (Sadler, 2004).

The *VKORC1* gene spans 5126 base pairs on human chromosome 16, contains three exons, and encodes a protein of 163 amino acids with a calculated relative molecular mass of about 18 kDa (Rost *et al.*, 2004a). There are several alternative splice variants and the cDNA encoding the protein with the full enzymatic activity and the complete gene sequence can be found at the NCBI web site under the designation AY521634 and AY587020, respectively.

Expression of human *VKORC1* in *Spodoptera frugiperda* (Sf9) cells and in *Pichia pastoris* results in enhanced VKOR activity over low endogenous constitutive levels. Sequence-based search methods reveal that human *VKORC1* belongs to a large family of homologous genes found in vertebrates, insects, plants, protists, archaea, and bacteria. All orthologs contain the same five completely conserved amino acids, including two cysteines found in a tetrapeptide motif that is presumably required for redox function (Li *et al.*, 2004; Oldenburg *et al.*, 2006). The conservation of these sequences across unrelated species underlines their important function and also suggests that *VKORC1* and VKOR activity arose very early in the development of eukaryotic life, probably with another function not directly related to γ -carboxylation of proteins (Robertson, 2004). What this function could have been remains enigmatic.

II. VKOR: IS IT A COMPLEX?

At its first description, VKOR was thought to be a large multiprotein complex. This notion became questionable when the expression of just one protein could confer VKOR activity on insect cells that lacked such activity (Li *et al.*, 2004). Apparently, this small protein of only 163 amino acids, and a relative molecular mass of about 18 kDa, was solely responsible for recycling vitamin K.

VKORC1 can be purified, for example, from ER membranes of mammalian cells (Cain *et al.*, 1997), but this procedure is difficult due to high hydrophobicity of the molecule. Recent data from *in vitro* translation and cotranslocation experiments demonstrated a three-transmembrane topology of VKORC1 (Tie *et al.*, 2005). The suggested structure can be summarized as follows: ER-lumenal N-terminus (with around 10 amino acids), three trans-membrane α -helices bracketing one large cytoplasmic loop (~69 amino acids) between the first and second transmembrane helices

and a small ER-luminal loop (~ 7 amino acids) between the second and third helices, and a cytoplasmic C-terminus (~ 16 aa) (Fig. 2.2).

Alignments of amino acid sequences of VKORC1 orthologs indicate several conserved amino acids and functional motives and have recently been reviewed by Oldenburg *et al.* (2006). For example, there are three conserved amino acids in the cytoplasmic loop (two cysteines, one at position 41 and one at 53, and there is always a Ser or Thr at position 57) and also two additional conserved cysteines (Cys132 and Cys135) that probably are buried in the ER membrane. These last two amino acids are contained in a CIVC (Cys, Ile, Val, and Cys) redox motif with an essential role in vitamin K reduction. This became clear from experiments in which Cys132 or Cys135 were mutated to Ser, which completely eliminated VKOR activity. It is important to note that there are no reports of natural mutations in these five conserved amino acids in human or rodents.

Just like the CIVC redox motif, the warfarin binding site of VKORC1 is predicted to be located into the lipid bilayer of ER membrane (Fig. 2.2). This notion is compatible with the fact that both warfarin and vitamin K are hydrophobic and preferably localize to a lipid environment (Wallin *et al.*, 2002). The physical adjacency of structural elements important for the VKORC1 warfarin interaction and vitamin K epoxide reduction suggests possible mechanisms for warfarin inhibition of VKOR activity (Pelz *et al.*, 2005).

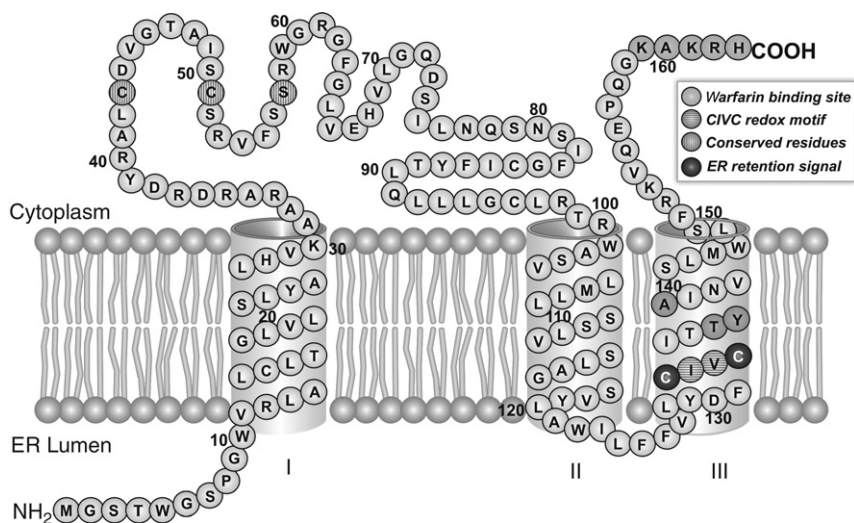


Figure 2.2 The three-transmembrane structure of vitamin K epoxide reductase complex subunit 1 (VKORC1). ER, endoplasmic reticulum; CIVC, cysteine-isoleucine-valine-cysteine amino acids. See details in the text. Adapted from Tie *et al.* (2005, Fig. 7, p. 16415).

Another important topological aspect is the close proximity of VKORC1 and GGCX in the ER membrane. This probably serves as a mechanism to limit the oxidation of nascent recycled vitamin K hydroquinone, once the ER lumen has an oxidative environment. However, it does not appear that VKORC1 and GGCX form a stable complex, because the two enzymes can be easily separated by mild chromatographic methods (Cain *et al.*, 1997).

Some authors (Cain *et al.*, 1997; Guenthner *et al.*, 1998; Rost *et al.*, 2004a; Wajih *et al.*, 2004) claimed that there are other components in the reductase system, such as microsomal epoxide hydrolase, glutathione-S-transferase, or calumenin. However, there was considerable evidence against this hypothesis. For example, a mouse in which microsomal epoxide hydrolase was knocked-out exhibited no abnormal phenotype (Miyata *et al.*, 1999). Also, it has been shown that calumenin, a member of the CREC (Cab-45, reticulocalbin, ERK-45, and calumenin) subfamily of Ca^{2+} -binding proteins, inhibits VKOR activity and confers warfarin resistance to VKOR in COS-1 cells (Wallin and Hutson, 2004). Finally, all known mutations that affect vitamin K-dependent carboxylation could be assigned to either VKOR or GGCX (Rost *et al.*, 2004b).

Nevertheless, because the active site of VKOR appears to include a canonical CIVC redox motif, one would expect that another enzyme is participating in the reaction. This expectation was based on findings in, for example, *Escherichia coli* where thioredoxin reductase (that reduces thioredoxin) completes the cycle of reduction. Further support for this hypothesis comes from studies in which it was noted that when lipoamide reductase, reduced thioredoxin, and protein disulfide isomerase (PDI) were added to microsomes, they all supported VKOR activity (Stafford, 2005).

Finally, a recent published study (Wajih *et al.*, 2007), with liver microsomal membrane proteins and using immunoprecipitation and two-dimensional SDS-PAGE techniques, demonstrated the existence of a VKOR enzyme complex where PDI and VKORC1 appear to be tightly associated subunits. PDI is a thioredoxin-like oxidoreductase and chaperone present at high concentration in the ER and provides electrons for reduction of the CXXC center in VKORC1. So, the energy required for γ -carboxylation of proteins is provided by dithiol-dependent oxidative protein folding in the ER and thus is linked to *de novo* protein synthesis (Fig. 2.3).

III. VKORC1 IN CLINICAL PRACTICE

Because the dose of warfarin required for a therapeutic level of anticoagulation varies greatly among patients, the utilization of this drug is accompanied by either a high risk of bleeding or of a thrombotic event, especially during the initial phase of the treatment. Therefore, tailoring

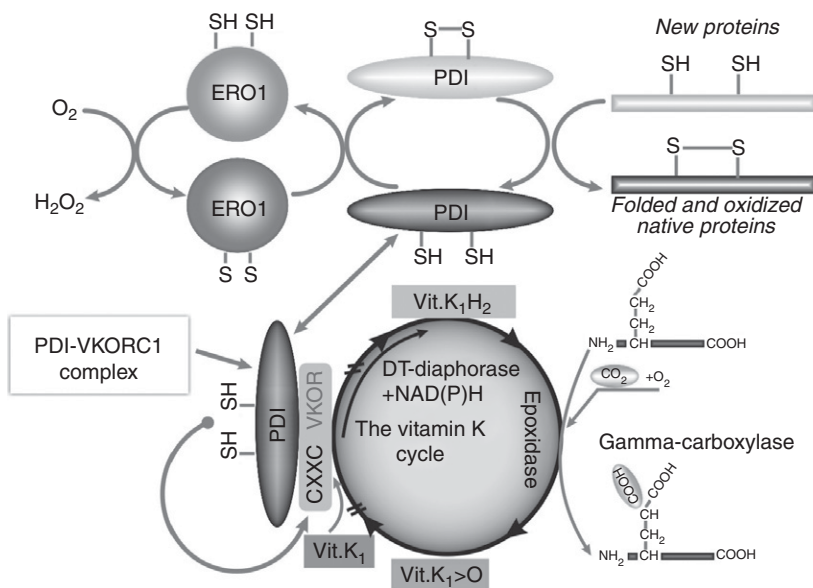


Figure 2.3 The hypothetical model of the protein disulfide isomerase (PDI) and vitamin K epoxide reductase complex subunit 1 (VKORC1) complex. PDI catalyzes dithiol-dependent oxidative folding of newly synthesized proteins in the ER, and it results in the reduction of its thioredoxin-related redox center CGHC (S-S is modified to SH-SH). Re-oxidation of the redox centers by endoplasmic reticulum oxidase1 (ERO1) is a major pathway for the continuous operation of PDI in oxidative folding. Some of the PDI in the ER may form stable complexes with VKORC1. The PDI-VKORC1 protein complex is responsible for reduction of vitamin K epoxide (vit. K > O) to form reduced vitamin K (vit. K₁H₂), the essential cofactor for γ -carboxylation of vitamin K-dependent proteins. Adapted from [Wajih et al. \(2007, Fig. 9, p. 2633\)](#).

therapy to reduce the side effects is appealing, especially as warfarin remains the drug of choice for anticoagulation treatment.

Warfarin commonly consists of a racemic mixture of *R*- and *S*-enantiomers, with the *S*-form being more active than the *R*-form ([Wadelius et al., 2005](#)). Both enantiomers block VKORC1 activity, and consequently vitamin K recycling.

Studies of the pharmacodynamics and pharmacokinetics of warfarin have revealed several environmental and genetic factors which may determine the variability of dose. Among the genetic factors, *VKORC1* gene seems to be the most relevant contributor to warfarin sensitivity. Pharmacogenetic studies have demonstrated a strong association between single nucleotide polymorphisms (SNPs) in this gene and the requirement of warfarin dosage ([D'Andrea et al., 2005](#); [Li et al., 2006](#); [Reitsma et al., 2005](#); [Rieder et al., 2005](#); [Wadelius et al., 2005](#)). It was demonstrated that

SNPs in *VKORC1* could reduce VKOR activity, like R98W, or result in warfarin resistance, like R58G, V29L, V45A, and L128R (Rost *et al.*, 2004a). The screening for *VKORC1* polymorphisms that evidently affect warfarin dosage could be helpful to tailor dosage at the onset of treatment.

In addition, part of the variability in a single patient's response to warfarin over time probably arises from variation in the dietary intake of vitamin K. It was already demonstrated that daily intake of vitamin K was indeed lower in patient with unstable anticoagulation compared to stably anticoagulated patient (Sconce *et al.*, 2005). It seems logical, therefore, that dosing patients with antagonist of vitamin K and vitamin K simultaneously should limit this variation. Indeed, a recent study suggested that daily supplementation of low doses of vitamin K in patients using phenprocoumon for anticoagulation treatment might be beneficial (Rombouts *et al.*, 2006). The problem with using vitamin K₁ plus warfarin is the possibility of arterial calcification, such as observed in rats (Price *et al.*, 1998). However, when the rats were fed warfarin and vitamin K₂, no arterial calcification was demonstrated (Spronk *et al.*, 2003). As known, vitamin K₂ is preferentially taken up in nonliver tissues, but it can be used by the liver to carboxylate the vitamin K-dependent proteins and levels of anticoagulation can be achieved, providing satisfactory therapeutic results.



IV. CONCLUSION

The description of the *VKORC1* gene, in 2004, provided an important step forward in understanding vitamin K metabolism. In the first place, this discovery is giving us detailed insight in the biochemical pathway involved in vitamin K cycling, including the putative role of PDI. In addition, the discovery of the *VKORC1* gene was important for understanding the wide variability in the individual response to vitamin K antagonists like warfarin. Several studies have shown that the activity of the VKORC1 enzyme is determined by genetic variants, which helps to explain interindividual and also interethnic differences in therapeutic response to coumarin drugs. On the basis of these results, it may become feasible to tailor treatment with vitamin K antagonists and limit the side effects of under- or over-anticoagulation.

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THE VITAMIN K CYCLE

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Abstract

Vitamin K is a collective term for lipid-like naphthoquinone derivatives synthesized only in eubacteria and plants and functioning as electron carriers in energy transduction pathways and as free radical scavengers maintaining intracellular redox homeostasis. Paradoxically, vitamin K is a required micronutrient in animals for protein posttranslational modification of some glutamate side chains to γ -carboxyglutamate. The majority of γ -carboxylated proteins function in blood coagulation. Vitamin K shuttles reducing equivalents as

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electrons between two enzymes: VKORC1, which is itself reduced by an unknown ER luminal reductant in order to reduce vitamin K epoxide ($K>O$) to the quinone form (KH_2); and γ -glutamyl carboxylase, which catalyzes posttranslational γ -carboxylation and oxidizes KH_2 to $K>O$. This article reviews vitamin K synthesis and the vitamin K cycle, outlines physiological roles of various vitamin K-dependent, γ -carboxylated proteins, and summarizes the current understanding of clinical phenotypes caused by genetic mutations affecting both enzymes of the vitamin K cycle. © 2008 Elsevier Inc.

I. INTRODUCTION

Vitamin K was discovered in the early 1930s by the Danish biochemist Henrik Dam and coworkers while studying dietary deficiency diseases resembling scurvy in chickens. When fed a fat- and cholesterol-free diet, the animals developed large subcutaneous and intramuscular haemorrhages. Compared to scurvy, this disease phenotype was not prevented by administering ascorbic acid and, thus, led to a search for a new, fat-soluble antihemorrhagic factor. In 1935, after experiments on several hundred chickens fed with several experimental diets depleted or enriched with various fractions of fat extracts or fat-soluble vitamins, Dam was able to describe the nature of this factor (Dam, 1935). These experimental results excluded the fat-soluble vitamins A, D, and E as the antihemorrhagic factor, so he proposed to name the new factor vitamin K (for Koagulation from the German and the Scandinavian languages). By carefully observing which foodstuffs alleviated the hemorrhagic condition, the fat-soluble vitamin was found to be enriched in hog liver fat and hemp seed extracts, but its chemical nature and structure remained elusive. Purification and crystallization of vitamins K1 and K2 took another four years (Almquist, 1936a,b; Binkley *et al.*, 1939; McKee *et al.*, 1939). Structure determination of vitamin K1 was performed in the lab of Edward Doisy (MacCorquodale *et al.*, 1939). For the discovery of vitamin K, Dam was awarded the Nobel Prize in Physiology or Medicine in 1943; together with Doisy for the purification, characterization, and synthesis.

Based on the work of Fritz (Dam *et al.*, 1936; Schönheyder, 1936), who identified inactive prothrombin as the cause of vitamin K deficiency-induced bleeding, Armand Quick was the first to draw a parallel between the sweet clover disease, a hemorrhagic disease in cattle fed on spoiled hay, and vitamin K deficiency (Quick, 1937). This later led to the discovery of dicoumarol in moldy silage, which is produced as a fungal metabolic product of coumarin, a mildly hepatotoxic substance found in many plants (Campbell *et al.*, 1940).

Use of vitamin K in medicine immediately followed its availability in pure form. At first, patients suffering from jaundice and biliary problems

leading to bleeding tendency were treated with vitamin K. Prophylactic treatment of newborns with vitamin K quickly became a main focus of therapeutic use to prevent excessive bleeding at birth and is still administered for this purpose today (Lehmann, 1944; Waddell and Guerry, 1939). At about the same time, based on the work of Link and Campbell, the vitamin K antagonist dicoumarol was subjected to clinical trials in Wisconsin General Hospital and the Mayo Clinic as an oral anticoagulant in prevention of venous and arterial thrombosis. It was for several years the most popular oral anticoagulant. While working to synthesize more potent vitamin K antagonists, Link in 1948 developed warfarin as a rodenticide. In 1951, first studies were undertaken to introduce warfarin as a therapeutic anticoagulant. It was found to be superior to dicoumarol, and in 1954 was approved for medical use. Despite their worldwide intensive use, the action of vitamin K antagonists remained unclear until 1974. At this time, Matschiner postulated a cyclic interconversion of vitamin K and vitamin K-epoxide, which could be inhibited by warfarin. Detailed analysis of this was published in 1978 by the lab of Suttie (Bell and Matschiner, 1970; Matschiner and Willingham, 1974; Matschiner *et al.*, 1970; Sadowski *et al.*, 1977; Whitton *et al.*, 1978; Willingham and Matschiner, 1974; Willingham *et al.*, 1976; Zimmermann and Matschiner, 1974). Another 26 years later, the complex biochemical relationship between vitamin K, its epoxide, and coumarins was enlightened by cloning the *VKORC1* gene, encoding a small enzyme that binds these substances and catalyzes the rate-limiting step of the vitamin K cycle. Genetically mutated variants have been shown to cause warfarin-resistance phenotypes as well as pathogenic deficiency of all vitamin K-dependent (VKD) coagulation factors (Li *et al.*, 2004; Rost *et al.*, 2004a).



II. VITAMIN K BIOSYNTHESIS

Vitamin K is a collective term for several related chemical compounds. They all share a 2-methyl-1,4-naphthoquinone backbone structure, but differ in the composition of the side chain at position C-3. Vitamin K1, first isolated from alfalfa and present in cyanobacteria and plants, possesses a mostly saturated, C-20 phytyl side chain (therefore called phyloquinone), while vitamin K2 from microbial organisms represents a group of compounds characterized by a partly unsaturated, predominantly C-40 side chain (menaquinone). In organisms producing vitamin K, both kinds are involved in electron transport processes: phyloquinone acts as the electron transfer cofactor A1 of photosystem I in plants, while menaquinone plays an essential role in several microbial electron transport systems.

A. Menaquinone

The biosynthesis of menaquinone has been extensively studied in *Escherichia coli* and *Bacillus subtilis*. Earlier, it has been demonstrated by isotopic trace experiments, that the naphthoquinone nucleus is built from seven carbon atoms derived from shikimate via chorismate and three remaining carbons derived from α -ketoglutarate (Bentley and Meganathan, 1982; Campbell *et al.*, 1967). Eight genes, *menA* through *menH*, were cloned and characterized (Meganathan, 2001) from eubacteria. These genes are often organized into operons encoding all enzymatic steps necessary for menaquinone biosynthesis (Meganathan, 2001; Taber *et al.*, 1981).

The biosynthetic pathway of menaquinone is initiated by isomerization of chorismate to isochorismate. This reaction is catalyzed by MenF (Daruwala *et al.*, 1996). MenD, through an addition reaction combining isochorismate and a succinic semialdehyde-thiamine PP_i (TPP) anion, produces 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC) (Palaniappan *et al.*, 1992). SHCHC is then dehydrated by MenC to yield the aromatic benzenoid *o*-succinylbenzoate (Sharma *et al.*, 1993), which is converted by MenE to a CoA thioester (Sharma *et al.*, 1996). The following cyclization of the naphthalene aromatic ring and thioesterolysis of the CoA is performed by sequential action of MenB and MenH, generating 1,4-dihydroxy-2-naphthoate (Meganathan, 2001; Sharma *et al.*, 1992). Finally, menaquinone is produced by prenylation and methylation, catalyzed by MenA and MenG (Koike-Takeshita *et al.*, 1997; Suvarna *et al.*, 1998).

In most anaerobic bacteria and in many aerobic Gram-positive bacteria, menaquinones serve as the sole quinone in the electron transport chain. In some facultative aerobic/anaerobic Gram-negative bacteria such as *E. coli*, both ubiquinone and menaquinone are used, the first during aerobiosis and the latter as an alternative electron carrier under anaerobic conditions (Meganathan, 2001). Furthermore, menaquinone is a cofactor of the photosynthetic reaction center of green sulfur bacteria (Hauska *et al.*, 2001; Kjær *et al.*, 1998) and has been detected in photosystem I of the red alga *Cyanidium caldarium* (Yoshida *et al.*, 2003).

B. Phylloquinone

1. Biosynthesis of phylloquinone in cyanobacteria

Phylloquinone with its C-20 phytyl chain at position C-3 differs only partially from the menaquinones with their unsaturated (mostly) C-40 side chains. This difference has been attributed to the MenA enzyme, serving as a phytyl transferase for phylloquinone and as a prenyl transferase for menaquinone (Johnson *et al.*, 2000). Nevertheless, synthesis of the naphthalene ring for both menaquinone and phylloquinone likely follows the same enzymatic steps. The biosynthesis of phylloquinone was studied in

detail in the cyanobacteria *Synechocystis* sp. PCC 6803. By comparison with genes encoding Men enzymes of the menaquinone biosynthetic pathway in *E. coli*, homologs for all genes were identified in *Synechocystis* (Johnson *et al.*, 2000, 2003; Sakuragi *et al.*, 2002). It was shown that separate knockouts of five of these genes resulted in complete loss of phyloquinone synthesis (Johnson *et al.*, 2000, 2003). These studies confirmed the Men pathway as the route of phyloquinone biosynthesis in cyanobacteria.

2. Biosynthesis of phyloquinone in plants

Compared to microorganisms, relatively little is known about phyloquinone biosynthesis in plants. Early work was done on the site of synthesis in spinach plants, where the principle reactions have been detected in the chloroplast (Schultz *et al.*, 1981). As in bacteria, isotopic trace experiments revealed chorismate and α -ketoglutarate as building blocks of the naphthoquinone nucleus. Enzymatic activity similar to MenA and MenG were identified in chloroplasts. Here, the methylation of the naphthoquinone nucleus takes place in the thylakoid fraction, while phytylation is performed in the chloroplast envelope membrane (Kaiping *et al.*, 1984; Schultz *et al.*, 1981). Recent cloning of a menA homolog in *A. thaliana* (Shimada *et al.*, 2005) suggests phyloquinone synthesis in plants is generally performed by the Men biosynthetic pathway.

III. VITAMIN K CYCLE

Although the impact of vitamin K on blood coagulation was already recognized in the mid-1930s by Dam and coworkers, a concrete mechanism for its action was unknown until the vitamin K cycle effecting posttranslational γ -carboxylation of proteins was discovered. Schönheyder (Dam *et al.*, 1936; Schönheyder, 1936) had already identified inactive prothrombin as the cause of vitamin K deficiency-induced bleeding, although nothing was known of mechanistic detail. In 1974, Stenflo (1974) and Stenflo *et al.* (1974) isolated a proteolytically cleaved aminoterminal heptapeptide from both active and warfarin-induced inactive prothrombin. By cyanogen bromide degradation, amino acid analysis, mass spectrometry, and NMR spectroscopy, they identified γ -carboxyglutamic acid (Gla) as a modified amino acid in normal prothrombin. Since Gla can bind Ca^{2+} with high affinity, while only unmodified glutamic acid is present in coumarin-induced inactive prothrombin, they concluded that Gla mediates binding of Ca^{2+} that is necessary for biological function of vitamin K dependent coagulation factors.

Four years earlier, in February 1970, Matschiner and Bell identified vitamin K-epoxide as a warfarin-induced metabolite of vitamin K in rat liver and heart (Matschiner *et al.*, 1970). In December of the same year,

reported on the procoagulant activity of vitamin K-epoxide, which again could be blocked by warfarin (Bell and Matschiner, 1970). This was the first evidence for vitamin K-epoxide reductase (VKOR) activity. Combining all these observations, a hypothesis of a cyclic conversion of vitamin K to vitamin K-epoxide and back to vitamin K, producing active coagulation factors and also inhibited by warfarin, was established. This was termed the “vitamin K cycle” (Bell and Matschiner, 1972). Although the procoagulant activity of vitamin K-epoxide was already observed by Fieser *et al.* (1941), the concept of a vitamin K cycle that could be blocked by coumarins was first established 30 years later.

With growing knowledge on the underlying biochemical pathways, two enzymes acting in this cycle were identified: (1) γ -carboxylase which modifies glutamic acid residues to Gla and (2) vitamin K-epoxide reductase which converts vitamin K-epoxide back to vitamin K.

A. γ -Glutamyl carboxylase

γ -Glutamyl carboxylase (GGCX) is an integral membrane enzyme located in the rough endoplasmic reticulum. Several proteins including coagulation factors II, VII, IX, X, osteocalcin, and matrix Gla protein (MGP) undergo VKD posttranslational modification by GGCX. Further insight into GGCX function was gained as Wu *et al.* (1991) cloned human GGCX and Kuo *et al.* (1995) mapped the GGCX gene to chromosome 2p12. Very recently, 2D crystals of GGCX were produced allowing structural investigation by electron crystallographic methods (Schmidt-Krey *et al.* 2007). A cryo-EM projection map at 12-Å resolution revealed that GGCX crystallizes as a monomer. Further studies are currently underway aimed at constructing a high-resolution model of the enzyme.

The reaction mechanism can be described as follows: in the first step, a conserved ~18-amino acid long peptide sequence upstream of a domain comprising several glutamic acid residues (Gla domain) binds to a propeptide recognition site (aa 495–513) within the γ -carboxylase (Furie *et al.*, 1999; Knobloch and Suttie, 1987; Lin *et al.*, 2002; Pan and Price, 1985; Price and Williamson, 1993). Initially, this sequence was thought to have a low affinity for binding the propeptide, blocking the enzyme’s active center and thus preventing promiscuous carboxylation of glutamates of proteins lacking a propeptide (Price and Williamson, 1993). Finally, its function was identified as the propeptide-binding site, not interacting with the active center (Lin *et al.*, 2002). Due to allosteric activation initiated by propeptide binding, transfer of the unmodified Gla domains to the active center is enhanced (Knobloch and Suttie, 1987). The residues between aa 393 and 404 have been shown to be of particular importance (Mutucumarana *et al.*, 2003). In turn, the propeptide binding causes a second allosteric conformational change, leading to an increased affinity of the γ -carboxylase for

vitamin K hydroquinone (Soute *et al.*, 1992; Sugiura *et al.*, 1997). The final carboxylation process begins with the abstraction of a hydrogen from a glutamic acid residue. The generated carbanion reacts with CO₂, resulting in γ -carboxylated glutamic acid (or Gla). In this reaction, vitamin K hydroquinone serves as an electron donor and is oxidized to vitamin K2,3-epoxide. During this process, the propeptide stays bound to the active center, while the Gla domain translocated, resulting in positioning of the next unmodified glutamic acid residue for modification. When most or all of the possible Gla domains on the propeptide have been carboxylated, affinity for the active center of GGCX is reduced, releasing the modified factor. Although propeptide binding seems to represent an essential step of γ -carboxylation, recently a class of proteins containing Gla residues, but lacking a propeptide sequence, was isolated from the venom of the marine cone snail *C. marmoreu* [GlaCrisp; (crisp = cystein-rich secretory protein), Hansson *et al.*, 2006]. Although other cone snail venom proteins do not have the usual aminoterminal propeptide, they possess a carboxyterminal consensus sequence constituting a new GGCX binding motive (Giese *et al.*, 2002). GlaCrisp, however, is not homologous with respect to this binding site. As GlaCrisp represents a member of the CRISP protein family also present in mammals (Brown *et al.*, 2005), it may be likely that further Gla proteins lacking propeptide or carboxyterminal GGCX-binding sequences might be discovered in higher animals.

B. Vitamin K-epoxide reductase

VKOR is an enzyme localized to the rough endoplasmic reticulum, the same cell compartment as for GGCX. VKOR is responsible for synthesis of vitamin K hydroquinone, which in turn is used as electron donor for γ -carboxylation of vitamin K dependent proteins by GGCX. During this process, vitamin K hydroquinone is oxidized to vitamin K-epoxide, which was for a long time thought to be a degradation product of vitamin K. Later, the procoagulant activity of vitamin K-epoxide under certain conditions, as well as accumulation of vitamin K-epoxide in warfarin-treated rats, led to the hypothesis of a recycling process beginning with the reduction of vitamin K quinone to vitamin K hydroquinone, followed by oxidation to vitamin K-epoxide and subsequent reduction to vitamin K quinone. Even though there is a very limited availability of vitamin K *in vivo* in higher animals, the recycling process provides enough reduced vitamin K to continually drive γ -carboxylation of VKD proteins. This process is blocked by coumarins, specific inhibitors of VKOR activity. In practice, this effect is used in oral anticoagulation for the treatment of thromboembolic disorders by controlled reduction of active VKD coagulation factors as well as in pest control by rodenticides, where by the same mechanism fatal bleeding at higher coumarin dosages is induced.

1. Cloning of *VKORC1*

Molecular identification of the *VKORC1* gene began in 1997 in a family with diagnosed autosomal recessive VKD clotting factor deficiency (VKCFD). In affected patients of this family, coagulation factor activities could be restored by oral supplementation with vitamin K. Vitamin K-epoxide levels in the patients' sera were found to concomitantly rise, suggesting a defect in VKOR enzyme activity. Although VKOR enzyme activity could be measured with a biochemical assay since 1970 (Bell and Matschiner, 1970), purification and subsequent sequence determination of this membrane protein was not tractable. For this reason, VKOR activity was thought to be performed by a multiprotein enzyme complex, very labile and resistant to purification.

Therefore, we chose a classical positional cloning approach to identify the genetic defect in our patients and, thus, identify one of the enzymes participating in VKOR activity. As VKCFD can also be caused by defects in *GGCX* (VKCFD1), we first excluded mutations in that gene and, furthermore, also in enzymes that were at that time thought to be other proteins of the VKOR complex (e.g., glutathion-S-transferase, DT-diaphorase, and microsomal epoxide hydrolase). We then identified a 20-megabase (Mb) region on chromosome 16p12-q21 by genome-wide linkage analysis and linked to VKCFD independently of *GGCX*. This disease phenotype was subsequently named VKCFD2. Combined with previous data from warfarin resistance in rat mapped to the corresponding locus on a different chromosome, the region in question was narrowed down to 4 Mb. We systematically screened the putative 129 genes in this region by sequencing DNA from two patients suffering from VKCFD2 and four additional patients diagnosed as warfarin resistant. From each patient, mutations were found in the same gene that had no annotated function in genetic databases. Extending over 5,126 base pairs (bp), this small gene comprises three exons, coding a protein of 163 amino acids. When expressed *in vitro*, a robust VKOR activity could be measured, which was inhibited by warfarin. Due to the possibility that VKOR activity was indeed mediated by a multienzyme complex, we named this gene Vitamin K-epoxide Reductase Complex subunit 1 (*VKORC1*). Additionally, recombinant expression of mutations found in VKCFD2 or warfarin-resistant patients led to reduced VKOR activity or warfarin resistance, respectively, in *in vitro* experiments proving functional correlation of *VKORC1* expression and VKOR activity (Rost *et al.*, 2004a).

At the same time, we identified the *VKORC1* protein by positional cloning, another group chose an siRNA knock-down approach and arrived at the same result. Based on our former mapping results, Stafford and coworkers systematically knocked down transmembrane genes of unknown function in the candidate region and tested for reduction of VKOR activity in the cell lines. Finally, they cloned the same gene as we did, exhibiting both warfarin sensitivity and VKOR activity when recombinantly expressed (Li *et al.*, 2004).

2. VKORC1 structure and function

Once the VKORC1 sequence was available, several conserved homologous and orthologous genes and pseudogenes were identified. Not only in vertebrates, where the vitamin K cycle is necessary for γ -carboxylation of VKD proteins, but also in bacteria, protists, and plants lacking GGCX activity, homologous genes were identified. They all share high sequence similarity and identification of VKORC1 homologs in bacteria indicates that VKOR activity might have arisen early in evolution and might accomplish other functions not related to γ -carboxylation.

Determination of the structure of the VKORC1 protein has been one of primary research goals since cloning. *In vitro* translation/cotranslocation experiments (Tie *et al.*, 2005) as well as electronic structure prediction suggest a topology comprising three α -helical transmembrane domains. Beginning with a short (~ 10 aa) ER-lumenal N-terminus, a large cytoplasmic loop (~ 69 aa) between the first and second transmembrane helix follows. The structure is continued by a small ER-lumenal loop (~ 7 aa) between the second and third helix and a small cytoplasmic C-terminus (~ 16 aa).

Looking in more detail at the amino acid sequence, four cysteine residues (aa 43, 51, 132, 135 of the human sequence) and one residue, which is either a serine or threonine (aa 57), were found to be completely conserved in all species analyzed. Therefore, they were inferred to be plausible components of VKORC1 active center. In three studies, the importance of these residues on VKOR activity was investigated by *in vitro* mutagenesis and recombinant expression. Concordantly, all studies revealed complete loss of VKOR enzymatic activity, when Cys132 and Cys135 were mutated to serine or alanine, respectively. These two vicinal cysteines have been proposed to form the VKORC1 redox center, as a CXXC motif characterizes the active center of redox enzymes from the thioredoxin family, which facilitate the reduction of other proteins by cysteine thiol–disulfide exchange. In 1992, Soute *et al.* suggested protein disulfide isomerase (PDI, a thioredoxin-like oxidoreductase) or thioredoxin as the actual reducing partner providing electrons for vitamin K-epoxide reduction, but thioredoxin was soon excluded by Preusch (1992). Recently, Wajih *et al.* (2007) again provided evidence for PDI as part of the vitamin K cycle. With reduced RNase as a substrate for oxidative protein folding, they could prove PDI-mediated vitamin K-epoxide reduction by VKORC1. Furthermore, knock down of PDI by siRNA resulted in greatly diminished VKORC1 activity. Therefore, the CXXC center of VKORC1 may play a central mechanistic role in the vitamin K cycle.

Located adjacent to the VKORC1 CXXC motif is a sequence previously identified as a warfarin-binding site in the coumarin-sensitive NADH quinone oxidoreductase (NQOR). Mutations in this small TYA motif (aa 139–141) were found to cause complete warfarin resistance *in vitro* and

in vivo, providing evidence for direct involvement of these residues in warfarin binding of VKORC1.

Within the large cytoplasmic loop, three further conserved residues were found. Of these (Cys43, Cys51, and Ser/Thr57), we could show that mutations cause dramatic decreases in VKOR activity, implying important roles in VKORC1 function (Rost *et al.*, 2005). Recently, Stafford and coworkers presented results somewhat differing from ours (Jin *et al.*, 2007). When changing Cys43 to alanine, the results were comparable, but in Cys51Ala (instead of our Cys51Ser mutation), they measured VKOR activity similar to the wild-type enzyme. Recognizing this discrepancy, they mutated both cysteins at position 43 and 51 to alanine simultaneously, resulting in 110% of the wild-type activity. Deletion of the entire region between cystein 43 to 51, a structure shown to form a loop closed by a disulfide bond, likewise resulted in an activity close to that of wild type. Comparing these findings with our results, two possibly correlatable differences can be observed. First, activity of some similar mutants differs greatly; second, the assay system used for enzyme activity measurement is not the same. Jin *et al.* used a system of purified and reconstituted VKORC1 from insect cells, previously described by this group (Chu *et al.*, 2006). Here, it was demonstrated that no other proteins likely involved in a VKOR complex were present and the authors therefore concluded that no additional proteins are required for VKOR activity. Actually, they showed even greater vitamin K turnover rates in purified and reconstituted VKORC1 compared to microsomal VKORC1 preparations. In our system, VKORC1 activity from mutants overexpressed in a mammalian cell system (HEK 293) were measured in microsomes without further purification, thus retaining all possible reaction or complex partners. Therefore, Cys43 and 51 might be implicated in some as yet uncharacterized *in vivo* function or regulation of VKOR activity or as a potential transmembrane electron transport system (Jin *et al.*, 2007) that might maintain redox conditions for the CXXC motif. It is clear, however, that the discussion of VKORC1 function as part of a multienzyme complex or as a single enzyme will likely only be clarified with future *in vivo* studies designed to address this question.

IV. PHYSIOLOGICAL ROLE OF VITAMIN K DEPENDENT PROTEINS

Vitamin K is a necessary participant in functional modification of proteins that are involved in coagulation, calcium metabolism, and other physiological processes. All of these proteins are posttranslationally modified by carboxylation of glutamic acid residues to form Gla. Without vitamin K, the carboxylation does not occur and the proteins that are synthesized are

not posttranslationally modified resulting in biologically inactive clotting factors. As observed by Dam, vitamin K deficiency leads to excessive bleeding. In all of these proteins, binding of Ca^{2+} ions via chelating Glas plays a central role in their binding to lipids in biomembranes and modulation of their physiological function.

A. Coagulation

The VKD blood coagulation factors consist of several proteins with 10–12 Gla residues at the N-terminus of the mature protein. Of these, the procoagulatory proteins prothrombin (factor II, FII), factor VII (FVII), factor IX (FIX), and factor X (FX) circulate in blood as mostly inactive serine protease zymogens. After secretion into the circulatory system plasma, two or three of the Gla residues bind a single Ca^{2+} ion, thus stabilizing the final tertiary structure of the protein (Furie *et al.*, 1979; Tai *et al.*, 1984). This process is associated with a conformational change, exposing a phospholipid membrane-binding site. In the event of an injury, the VKD coagulation is triggered by a protein complex consisting of tissue factor and FVII. In this tissue factor pathway (formerly known as the extrinsic pathway of blood coagulation), the first step is binding of tissue factor to activated FVII (FVIIa), which is circulating at minimal concentrations in blood. By activating FIX and FX, the coagulation cascade proceeds. The main role of this tissue factor pathway is to generate a “thrombin burst,” thrombin being the most important constituent of the coagulation cascade in terms of its feedback activation role. In this pathway, the Gla-modified membrane-binding sites of FII, FVII, FIX, and FX play a central role. After binding to phospholipid surfaces, released by damaged cells or derived from platelets, the noncovalent binding is mediated by Ca^{2+} ions coordinated to both the surface-exposed Gla side chains on the clotting factors and to anionic lipids of the cell surface membrane. Through the coordination of Ca^{2+} ions, this interaction is tightened. This Ca^{2+} -mediated process is crucial to forming the tenase and prothrombinase complex of the tissue factor pathway.

In the absence of Gla residues or even when inefficient carboxylation results in fewer than the possible 10–12 modified residues, VKD clotting activity is dramatically reduced. Due to either hereditary conditions (mutations in VKORC1 or GGCX) or to acquired pathophysiological conditions (vitamin K deficiency due to malnutrition/malabsorption, coumarin poisoning, or autoimmune response to VKORC1/GGCX), a severe bleeding phenotype is observed. In some cases, especially due to acquired pathophysiology, high doses of vitamin K can reverse the bleeding phenotype. Although two VKD anticoagulant factors—protein C and protein S—are similarly affected by vitamin K deficiency too, the global procoagulant effect of vitamin K prevails.

B. Calcium homeostasis

As for coagulation factors, binding of Ca^{2+} ions is a required function of Osteocalcin and MGP. Both regulate calcium metabolism, Osteocalcin chiefly in bone and MGP predominantly in the extracellular matrix of soft tissue.

1. Osteocalcin

Osteocalcin is an abundant noncollagenous bone matrix protein (BMP) that is synthesized by osteoblasts and odontoblasts. Induced by Vitamin D3, this small, 49-amino acid protein is associated with the mineralized matrix of bone. Its function is absolutely dependent on three residues of Glu at positions 17, 21, and 24. This Glu domain forms an α -helix in which all Glu residues are surface-exposed and lie of the same face of the α -helix (Dowd *et al.*, 2003). This orientation enables osteocalcin to very tightly bind calcium within the hydroxyapatite crystal lattice. Furthermore, the carboxyterminal sequence of osteocalcin possesses recruitment activity for osteoclast progenitor cells, which are involved in bone resorption (Lian *et al.*, 1984). All these properties indicate that osteocalcin plays a focal regulatory role in the mineralization and remodelling of bone tissue. Consequently, numerous studies have shown that concentration of undercarboxylated (nonfunctional) osteocalcin (ucOC) is inversely correlated with bone mineral density (BMD) (Knapen *et al.*, 1998) and, furthermore, positively associated with bone fracture rate (Vergnaud *et al.*, 1997).

Most recently, it was reported that osteocalcin acts as a hormone. Karsenty and coworkers presented evidence that osteocalcin causes pancreatic beta cells to proliferate and release elevated levels of insulin (Lee *et al.*, 2007). Independently, a concomitant enhanced release of adiponectin from adipocytes was observed, leading to an increased sensitivity to insulin (Lee *et al.*, 2007). As osteocalcin knockout mice were found to be abnormally fat and prone to diabetes (Ducy *et al.*, 1996; Lee *et al.*, 2007), new functions of osteocalcin in metabolism have been inferred that might represent a promising new approach in diabetes research and therapy.

2. Matrix Gla protein

MGP is an extracellular matrix protein with strong Ca^{2+} -binding properties. Although initially isolated from bovine bone, MGP is expressed in almost all soft tissues with highest levels of expression found in vascular tissue, heart, lung, kidney, and cartilage (Fraser and Price, 1988), where MGP serves as a central inhibitor of tissue calcification. This function was clearly revealed by gene deletion experiments in mice, where mice lacking MGP were normal at birth but rapidly developed severe arterial calcification of the elastic lamellae and died from aortic rupture at the age of 2 months (Luo *et al.*, 1997). The most plausible explanation of the mechanism of action of MGP on soft tissue calcification might be sequestration of Ca^{2+} in nucleating hydroxyapatite,

inhibiting crystal growth (Price *et al.*, 1983). Consequently, restoration of MGP expression in arteries of MPG-knockout mice rescued the arterial mineralization phenotype, whereas expression of MGP in osteoblasts prevents bone mineralization (Murshed *et al.*, 2004). In humans, functional defects in MGP result in Keutel syndrome, a rare autosomal disorder characterized by midfacial hypoplasia and ectopic abnormal calcification (Munroe *et al.*, 1999). Due to its dependency on adequate γ -carboxylation of glutamate residues for full functionality, acquired deficiency of MGP, especially through prenatal exposure to coumarins, can result in a phenotype similar to Keutel syndrome.

C. Vitamin K and osteoporosis

The interaction between dietary vitamin K intake and bone health has been investigated in several epidemiological and interventional studies. In the *Framingham Heart Study*, serum levels of phylloquinone and menaquinone were shown to represent a good marker for osteoporotic fracture risk. It was shown that elderly men and women in the highest quartile of phylloquinone intake (median 254 $\mu\text{g}/\text{day}$) had a significantly lower risk of hip fracture than those in the lowest quartile of intake (median 56 $\mu\text{g}/\text{day}$) (Booth *et al.*, 2000). Furthermore, low dietary phylloquinone intake has been associated with low BMD (Booth *et al.*, 2003) and high levels of undercarboxylated serum osteocalcin (Collins *et al.*, 2006). In contrast, high intake of vitamin K was reported to decrease serum ucOC, sustaining lumbar BMD and prevent fractures in patients with osteoporosis (Miki *et al.*, 2003; Shiraki *et al.*, 2000). Another study investigating the combined effects of vitamins D and K demonstrated an additive beneficial effect in improving bone health when adequate levels of both vitamins were administered (Iwamoto *et al.*, 2003).

As a result, all studies commonly indicated that the amount of vitamin K needed for optimal γ -carboxylation of osteocalcin is significantly higher than the doses provided through recommended dietary intake alone (WHO recommendation 80 $\mu\text{g}/\text{day}$ or 1 $\mu\text{g}/\text{kg}$, respectively). Therefore, it is discussed to increase current dosage recommendations for vitamin K with regard to bone mineralization and risk of osteoporosis.



V. CLINICAL PHENOTYPES RELATED TO THE VITAMIN K PATHWAY IN MAN

A. Coumarin-based anticoagulation

Coumarins are the most frequently used oral anticoagulation medications for prevention and therapy of thromboembolic conditions. By 1954, warfarin became the most often prescribed drug globally. Despite its easy oral

application, warfarin along with other coumarin drugs exhibit some shortcomings. Most importantly, the therapeutic window for anticoagulation is relatively narrow. Together with a broad variation in individual drug requirement, this results in a relatively high incidence of bleeding complications such as intracerebral bleeding or re-thrombosis, especially in the first months of treatment (Kuijjer *et al.*, 1999; Palareti *et al.*, 1997). Although known factors such as weight, gender, age, and race influence the optimal individual dosage (Kamali *et al.*, 2004; Wilkinson and Sainsbury, 2003), the predicted and the actual optimal dose were not well correlated in many patients. A rationalization for this discrepancy is presented in the following two sections.

1. Coumarin sensitivity

Metabolism of most coumarins is primarily dependent on hepatic-localized hydroxylation by CYP2C9, one of the most abundant enzymes of the CYP2C family (Lapple *et al.*, 2003). For this enzyme, numerous polymorphisms and haplotypes have now been identified (homepage of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee: <http://www.cypalleles.ki.se>; accessed 30/08/2007), some showing wild-type activity, but several variants with greatly impaired enzymatic function complicate oral anticoagulation. In today's clinical practice, CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) which possess residual activity of 50–70% and 10%, respectively, play a major role. These defect alleles show a prevalence of around 15% (CYP2C9*2) and 7% (CYP2C9*3) in Caucasians. In patients from Africa or Asia, observed frequency of CYP2C9*2 and *3 is lower, but other defect alleles such as CYP2C9*11 has been shown to cause warfarin sensitivity in almost the same manner. Regarding prevalence, a calculated fraction of roughly 3% of patients with Caucasian ancestry will be homozygous or compound heterozygous for these alleles, predisposing them for over-anticoagulation. However, in spite of accurate knowledge of variant CYP2C9 alleles associated with impaired coumarin metabolism and other influencing factors, interindividual differences in drug requirement have been explained only partially (Aithal *et al.*, 1999; Higashi *et al.*, 2002; Visser *et al.*, 2004).

This problem was largely solved with the cloning of *VKORC1* and identification of this enzyme as a molecular target of coumarins (Li *et al.*, 2004; Rost *et al.*, 2004a). D'Andrea *et al.* (2005) shortly thereafter found an intronic polymorphism associated with greatly reduced coumarin dose requirement. This polymorphism was shown to be part of a complex haplotype, with each allele responsible for 25% dose reduction (Geisen *et al.*, 2005; Rieder *et al.*, 2005). In this haplotype VKORC1*2, a single variation in the promoter (VKORC1 c.1–1639G > A, rs9923231) leads to halved mRNA expression and subsequently reduced VKOR enzyme activity (Rieder *et al.*, 2005; Yuan *et al.*, 2005). Until now, several studies

have confirmed the correlation of the *VKORC1**2 haplotype and low dose coumarin requirement. In the same way, this haplotype also explains interethnic differences in coumarin dosage. While the dose-reducing haplotype *VKORC1**2 is highly prevalent in patients of Asian origin where frequencies up to 95% can be observed, *VKORC1**2 is rare in Africans (15%). In Europeans, a more balanced distribution of dose-reducing and dose-elevating haplotypes is seen (40% *VKORC1**2). Due to this circumstance, average coumarin dose requirements are low for Asians, intermediate for Europeans and high for Africans.

In light of the effects of various *VKORC1* and *CYP2C9* haplotypes, several clinical dosing algorithms were established combining genotype and other dose-influencing factors such as age, gender, and weight (Carlquist *et al.*, 2006; Millican *et al.*, 2007; Sconce *et al.*, 2005a,b; Tham *et al.*, 2006). Preliminary small clinical studies have been conducted (Carlquist *et al.*, 2006; Sconce *et al.*, 2005b). These report *VKORC1* genotype as the major predictor of coumarin dose, with *CYP2C9* genotype an important contributing modulator. Additionally, combination of low-dose alleles in both *VKORC1* and *CYP2C9* were discovered as strong indicators for high risk of severe over-anticoagulation (Quteineh *et al.*, 2005; Reitsma *et al.*, 2005; Schalekamp *et al.*, 2006). Therefore, premedication genotyping can now reveal patients with combinations of risk alleles, thus preventing overdosing and over-anticoagulation especially during the initial phase of dosage adjustment.

2. Coumarin resistance

Coumarin resistance is a rare, but potentially severe phenotype in some patients who are put on anticoagulation therapy. In order to achieve manageable long-term therapeutic effects, initial heparin anticoagulation for patients suffering from venous or arterial thromboembolic events is switched to coumarin-based anticoagulation over the long term. In some of these patients, standard coumarin dosing does not lead to the desired effect of prolonged clotting time. In the majority of these cases, combined homozygous *VKORC1* and *CYP2C9* wild-type alleles were identified as the cause of an elevated (often doubled) dosage requirement. In some rare cases, heterozygous mutations in *VKORC1* result in observed partial to complete resistance (Bodin *et al.*, 2005; D'Ambrosio *et al.*, 2007; Geisen *et al.*, 2005; Harrington *et al.*, 2005; Loebstein *et al.*, 2007; Rost *et al.*, 2004a). Typically for these patients, coagulation factor activity is not affected by these heterozygous mutations when anticoagulants are not administered. However, upon administering coumarin therapy, the functional allele is inhibited, but warfarin insensitivity of the mutant allele results in therapeutic doses requiring from 70 up to 250 mg warfarin per week (Rost *et al.*, 2004a). In single cases, complete resistance can be observed (greater than 250 mg dose per week has little effect).

A closer look at the mutations reveals some interesting trends. All, even those found in rat, cluster into two protein primary sequence domains (Pelz *et al.*, 2005). The first domain includes the third transmembrane alpha helix which harbors the postulated warfarin-binding motif TYA at amino acids 138–140 (Myszka and Swenson, 1991) and the CXXC redox-motif at amino acids 132–135 (Rost *et al.*, 2005; Wajih *et al.*, 2005). Mutations in this region lead to an abolished warfarin binding, resulting in an almost complete anticoagulant resistance. The consequences of mutations within the second mutation cluster, mostly located in the large extramembraneous loop, are more difficult to explain (amino acids 31–100). As discussed below, experimental data from several recent studies are in disagreement as to whether specific amino acid residues in the loop domain actually affect VKOR catalytic activity. To briefly summarize these results, although we found Cys43, Cys51, and Ser57 essential for VKORC1 activity (Rost *et al.*, 2005), Jin *et al.* (2007) provided evidence for enhanced VKOR activity in a construct lacking aa 43–51. Differences in the experimental systems were noted as a possible explanation for the discrepancies between the results. If, nevertheless, we assume that mutations in the loop can affect either VKOR enzymatic activity or warfarin inhibition, then we must postulate an “action at a distance” mechanism as the loop domain appears to be physically distant from the CXXC redox and warfarin-binding motifs. Verification or rejection of this postulate should be possible through interpretation of future functional studies and high-resolution structural models.

3. Perspectives of coumarin therapy

Recently, due to recent research results concerning molecular function of the vitamin K cycle and its inhibition by coumarins, considerations for safer anticoagulation therapy regimes by concomitant administration of vitamin K with coumarins have gained attention (Oldenburg, 2005). Although concomitant application of agonist and antagonist would seem to be contraindicated, several reasonable considerations have led to a new principle in oral anticoagulation. As previously stated, the main complication in oral anticoagulation is bleeding due to severe over-anticoagulation. In many cases, varying vitamin K dietary intake might be a contributing cause. As VKOR activity is directly dependent on vitamin K concentration and as the human body does not possess major vitamin K reservoirs, variable vitamin K intake will influence the efficiency of vitamin K recycling. As a result, coumarin dose is not only dependent on fixed variables such as genotype, age, and gender, but also variables over time such as vitamin K intake or initiation/termination of other drug therapies during anticoagulation therapy. Supplementing patients with low-dose vitamin K, not sufficient for reduction by NQO1, but adequate to supply warfarin-inhibited VKORC1 with a slightly elevated vitamin K concentration sufficient to enhance vitamin K cycling by mass action, should uncouple intrapersonal

anticoagulation dosing from nutritional habit. This hypothesis is strengthened by three recent publications. In healthy volunteers undergoing oral anticoagulation, substitution of 150 μg vitamin K per day did not change the International normalized ratio (INR) significantly (Schurgers *et al.*, 2004). Secondly, a significantly lower intake of vitamin K (even below the recommendations of the WHO in healthy persons) was observed in patients with unstable INR compared to patients with stable INR (Sconce *et al.*, 2005a). Finally, a first, small-scale clinical study, testing concomitant application of warfarin and low-dose vitamin K points towards a greater stability in anticoagulation for the vitamin K group, but cohorts were too small to draw final conclusions (Sconce *et al.*, 2007). Based on these observations, one could speculate that low-dose vitamin K in the range of WHO recommendation, for example, 80 μg per day, could stabilize INR values within the therapeutical range and thus reduce the risk of bleeding complications due to oral anticoagulation therapy.

Another important milestone in oral anticoagulation was released to the public on August 16, 2007 when the FDA changed the label for warfarin (see <http://www.fda.gov/cder/drug/infopage/warfarin/qa.htm>, accessed August 30, 2007) in order to make clinicians aware of the consequences of polymorphisms in *CYP2C9* and *VKORC1*. Although no general genetic testing for the *VKORC1**2, *CYP2C9**2, and *CYP2C9**3 alleles prior to warfarin therapy is mandatory, this change indicates that genotype-based individualized dose adaptation should become routine practice in the near future. An additional precedent has been set by the appearance of commercially available reagents for the analysis of pharmacogenetic relevant polymorphisms in *VKORC1* and *CYP2C9*.

B. Vitamin K-dependent clotting factor deficiency

Deficiency of VKD coagulation factors is a rare autosomal recessive bleeding disorder caused by mutations in *GGCX* (VKCFD1) or in the *VKORC1* gene (VKCFD2). Until now, several different *GGCX* mutations in 10 patients have been detected (Brenner *et al.*, 1998; Mousallem *et al.*, 2001; Rost *et al.*, 2004b, 2006; Spronk *et al.*, 2000; Vanakker *et al.*, 2007). In some patients, coagulation factor activity was reverted to normal by administration of 5 mg vitamin K per day, while the phenotype in other patients was only reversed partially, even when receiving 10 mg vitamin K daily (Brenner *et al.*, 1998; Mousallem *et al.*, 2001; Rost *et al.*, 2004b, 2006; Spronk *et al.*, 2000).

In two patients originating from Lebanon and Germany suffering from VKCFD, no mutation in *GGCX* could be detected (Fregin *et al.*, 2002; Oldenburg *et al.*, 2000). As described above, this led to discovery of *VKORC1* (Rost *et al.*, 2004a). In these patients, an identical single homozygous mutation is the cause of the observed VKD coagulation factor deficiency. Unlike VKCFD1 caused by mutations in *GGCX*, the

phenotype of VKCFD2 could be generally reversed completely in all patients by administration of comparatively low doses of vitamin K (~5–10 mg/week).

Comparing VKCFD1 and VKCFD2, two different mechanisms result in reduced coagulation factor activity. Mutations in *GGCX* were thought to target the propeptide or alternatively the vitamin K hydroquinone-binding site. While mutations in the propeptide-binding site of *GGCX* will lower the affinity of noncarboxylated VKD proteins or result in faster release of partially carboxylated substrates, mutations in the vitamin K-binding site might result in an increased dissociation constant for vitamin K hydroquinone, leading to a requirement for reduced vitamin K (Spronk *et al.*, 2000). Depending on the impairment of *GGCX* functionality, the phenotype can be reversed partial or completely by supplementation with large amounts of vitamin K.

Thus far, only one *VKORC1* mutation was found to cause VKCFD2. Here, reduction of vitamin K-epoxide is impaired (Rost *et al.*, 2004b), resulting in low concentrations of vitamin K hydroquinone which is required to drive gamma carboxylation. Due to the underlying difference from *GGCX*-mediated pathologies, reversal of the phenotype by administration of vitamin K has a different cause than for defects in *GGCX*. In liver, two pathways have been described for reduction of vitamin K to vitamin K hydroquinone. The first, performed by *VKORC1*, is highly inhibited by coumarin-based drugs, uses vitamin K-epoxide as well as vitamin K as substrate, and functions at low substrate concentrations (has high affinities for the substrates). The second pathway is via the microsomal enzyme NAD(P)H quinone dehydrogenase 1 (NQO1, DT diaphorase). This pyridine nucleotide-dependent enzyme is a two electron reductase that detoxifies several quinones derived from the oxidation of phenolic metabolites. At nonphysiological elevated concentrations, vitamin K quinone, but not vitamin K-epoxide, is a substrate for NQO1. Therefore, in patients suffering from VKCFD2, supplementation with vitamin K leads to reversal of the bleeding phenotype with accumulation of VKO in plasma (Rost *et al.*, 2004a; Oldenburg *et al.*, 2007). As NQO1 is less sensitive to warfarin compared to *VKORC1*, the antidotal effect of high dose vitamin K in warfarin intoxication can be attributed to NQO1 activity (Wallin, 1989).

C. Pseudoxanthoma elasticum-like phenotypes

Very recently, mutations in *GGCX* have been recognized as responsible for pseudoxanthoma elasticum (PXE)-like phenotypes (Vanakker *et al.*, 2007). PXE is an autosomal recessive disease affecting eyes, skin, and the arterial system. Mutations in the *ABCC6* gene, encoding an ATP-dependent transmembrane transporter were found to be causative in most, but not all patients. Vanakker *et al.* has described six patients without mutations in

ABCC6 exhibiting excessive skin folds and reduced VKD coagulation factor activity. Upon further investigation of these patients, several new mutations in *GGCX* were detected. As clotting factor activity was reduced to about 50% with a bleeding tendency in only two patients, they initially were diagnosed with PXE due to mild retinopathy and severe skin abnormalities (yellowish papules and generalized excessive and leathery skin folds). In skin biopsies surveyed by electron microscopy, differences from typical PXE morphologies were obvious. Although PXE-like calcifications were found, calcifications were restricted to limited areas and organized as electron-dense crystal-like precipitates. This finding is now attributed to undercarboxylated Gla proteins, most probably the MGP. In some, but not all, patients with apparent severe VKCFD1, similar signs of skin abnormalities have been observed. As PXE itself exhibits a heterogeneous appearance, one can speculate on additional hereditary factors that may modulate the phenotype beside mutations in *GGCX* and *ABCC6*.

D. Acquired forms of vitamin K deficiency

In some cases, a VKCFD phenotype can be explained by acquired factors that are not genetically based. Predominantly, malnutrition of long duration with low vitamin K intake results in diminished coagulation factor activity without severe medical consequences. However, during pregnancy, the effect of vitamin K-deficient nutrition or malabsorption due to antibiotic treatment or a partially removed ileum is more severe (Eash *et al.*, 2003; Sheffield *et al.*, 1976; Menger *et al.*, 1997; Nivelon-Chevallier, 1998). In these very rare cases, a phenotype of the newborn close to warfarin-induced embryopathy has been observed (Hall *et al.*, 1980; Harrod and Sherrod 1981). In both cases, the mechanism is the same. Due to reduced γ -carboxylation of osteocalcin and MGP, chondral structures of the newborn are calcified or bones are overall calcium deficient. Interestingly, a more severe phenotype was found for Keutel syndrome, where mutations in MGP were shown to result in abnormal cartilage calcification, peripheral pulmonary stenosis, and midfacial hypoplasia (Munroe *et al.*, 1999). Undercarboxylated MGP appears to be the primary influence for this phenotype.

VI. CONCLUSIONS AND PERSPECTIVES

The identification of VKORC1 has greatly improved understanding of the vitamin K cycle. It represents the major—or may be even the sole—component of the VKOR and the molecular target of coumarin. Just three years after detection of VKORC1, the translation of basic research in this field into clinical practice is progressing fast and already has led to concrete

perspectives of a safer individualized oral anticoagulant therapy by testing for VKORC1 variants and the application of low-dose vitamin K in addition to coumarins. Moreover, other downstream pathways of the vitamin K cycle as calcium homeostasis in osteoporosis and arterogenesis have been illuminated by VKORC1, thus stimulating and increasing the research activities in these fields.

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STRUCTURE, FUNCTION, AND MECHANISM OF CYTOSOLIC QUINONE REDUCTASES

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Abstract

Quinone reductases type 1 (QR₁) are FAD-containing enzymes that catalyze the reduction of many quinones, including menadione (Vit K₃), to hydroquinones using reducing equivalents provided by NAD(P)H. The reaction proceeds with a ping-pong mechanism in which the NAD(P)H and the substrate occupy alternatively overlapping regions of the same binding site and participate in a double hydride transfer: one from NAD(P)H to the FAD of the enzyme, and one from the FADH₂ of the enzyme to the quinone substrate. The main function of QR₁ is probably the detoxification of dietary quinones but it may also contribute to the reduction of vitamin K for its involvement in blood coagulation. In addition, the same reaction that QR₁ uses in the detoxification of quinones, activates some compounds making them cytotoxic. Since QR₁ is elevated in many tumors, this property has encouraged the development of chemotherapeutic compounds that become cytotoxic after reduction by QR₁. The structures of QR₁ alone, and in complexes with substrates, inhibitors, and chemotherapeutic prodrugs, combined with biochemical and mechanistic studies have provided

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invaluable insight into the mechanism of the enzyme as well as suggestions for the improvements of the chemotherapeutic prodrugs. Similar information is beginning to accumulate about another related enzyme, QR2. © 2008 Elsevier Inc.

I. INTRODUCTION

Some cancer protective agents work by the induction of phase II detoxification enzymes. One such enzyme, quinone reductase (QR, QR1, or NQO1; NAD(P)H:quinone acceptor oxidoreductase; EC 1.6.99.2, also called DT-diaphorase and vitamin K reductase), is a widely distributed cytosolic flavoprotein that catalyzes the obligatory two-electron reduction of quinones (Iyanagi, 1987), quinone imines, and azodyes. Quinone reductase type 1 (QR1) protects cells from the mutagenic and carcinogenic effects of quinones and related compounds (Dinkova-Kostova and Talalay, 2000; Huggins and Pataki, 1965; Talalay *et al.*, 1987) by diverting quinones from both redox cycling (with generation of reactive oxygen species) and direct reaction with intracellular sulfhydryl groups. The same enzyme, which is elevated in many tumors, has also been implicated in the reductive activation of chemotherapeutic quinones such as mitomycins (Pan *et al.*, 1995). Thus, the same catalytic activity that protects normal cells from quinones activates chemotherapeutic drugs to cytotoxic compounds that lead to the death of cancer cells. Taking advantage of this activity, a large number of cancer chemotherapeutic prodrugs are being developed using QR1 as the activating target. In addition, QR1 is exquisitely sensitive to inhibition by dicoumarol and related anticoagulants and may play a critical role in the generation of reduced vitamin K required for the γ -carboxylation of glutamate residues in certain blood coagulation proteins (Wallin and Hutson, 1982).

Reviews of the discovery, purification, molecular characteristics, induction, and other properties of this enzyme have been published (Ernster, 1987; Talalay *et al.*, 1987). It is known (Bayney *et al.*, 1987; Ernster, 1987; Haniu *et al.*, 1988; Prochaska, 1988; Robertson *et al.*, 1986) that QR1 is a dimer formed by two identical subunits of 273 amino acids each; that the FAD prosthetic group in each subunit is noncovalently attached but remains bound during catalytic cycling; that NADH or NADPH cycles in and out of the enzyme to provide the reducing equivalents; that NAD(P)⁺ must be released from the enzyme before substrate can bind; that no products of one-electron reactions have ever been observed during catalysis with QR1. Structural information obtained during the last two decades has helped rationalize existing information and has shed significant light on the most important questions in this system. Structures of rat, mouse, and human QR1s (Amzel *et al.*, 1986;

Prochaska, 1988; Prochaska and Talalay, 1986; Prochaska *et al.*, 1987; Ysem and Prochaska, 1989) have been determined, as well as the structures of complexes of these enzymes with substrates, cofactors, and inhibitors (Faig *et al.*, 2000; Li *et al.*, 1995). Based on these structures and the biochemical and kinetic information, a mechanism for the two-electron reduction of quinones by QR (Li *et al.*, 1995) was proposed. The structures of QR1 complexes with selected chemotherapeutic compounds (Faig *et al.*, 2001; Skelly *et al.*, 1999) were used to suggest empirical rules for the improvement of these prodrugs.

Another cytosolic quinone reductase, QR2, was identified and characterized during the 1990s (Zhao *et al.*, 1997) and was found to be identical to an enzyme described in 1961 (Liao *et al.*, 1962) as a flavoprotein that catalyzed the oxidation of reduced nicotinamide. The possible functions of QR2 have been reviewed recently (Vella *et al.*, 2005). This enzyme can use *N*-ribosyl- and *N*-alkyldihydronicotinamides as the source of reducing equivalent but not by NADH, NADPH, or NMNH (reduced nicotinamide mononucleotide). QR2 is highly similar to QR1 although it is a smaller protein. The structure of human QR2 and of its several complexes, including that of menadione (Vit K3, a vitamin K precursor), has also been determined (Buryanovskyy *et al.*, 2004; Foster *et al.*, 1999; Fu *et al.*, 2005).

In the following sections, description and discussion of the better understood QR1 is followed by a brief review of available information on QR2.

II. QUINONE REDUCTASE TYPE 1

A. Physiological importance of QR1

1. Protective functions of QR1

It has been shown that (a) QR1 is a major xenobiotic metabolizing enzyme, (b) its induction is associated with protection against the toxic and carcinogenic effects of many electrophiles, and (c) induction of QR1 is frequently coordinated with other phase 2 detoxification enzymes (e.g., glutathione transferases) that also promote the inactivation of electrophiles (Dinkova-Kostova and Talalay, 2000; Talalay *et al.*, 1987, 1988). The following is a partial list of that evidence:

- The ability of compounds to protect against the toxicity of quinones correlates extremely well with their ability to induce QR (Huggins and Pataki, 1965; Talalay *et al.*, 1987).
- Inhibition of QR dramatically enhances the toxicity of quinones (Galaris *et al.*, 1985; Thor *et al.*, 1982).
- Elevation of QR in postmitochondrial supernatant fractions of mouse liver cytosols suppresses singlet oxygen chemiluminescence generated by oxidative cycling of menadione (Prochaska *et al.*, 1987).

- QR, like other detoxification enzymes, is elevated in preneoplastic hepatic nodules believed to arise from selection of cells that tolerate the cytotoxic carcinogens (Roomi *et al.*, 1985).
- Direct addition of QR reduces the toxic quinone metabolites of benzene generated by liver fractions (Smart and Zannoni, 1984). Similarly, the toxicity of polycyclic aromatic hydrocarbons is reduced by QR and enhanced by the potent inhibitor, dicoumarol (Swanson *et al.*, 1986).
- Entrapment of purified QR into human erythrocytes reduces the toxicity of menadione (lowers the extent of methemoglobin formation) (Benatti *et al.*, 1987).
- QR plays a major role in reducing chromium (VI), thereby reducing its strong mutagenicity (De Flora and Ramel, 1988).
- QR plays a protective role against the induction of renal tumors by diethylstilbestrol in the hamster (Roy and Liehr, 1988).
- Development of resistance to menadione-induced oxidative damage by several cell lines is associated with an elevation in QR activity (Chiou *et al.*, 1999).
- QR1-deficient individuals exposed to occupational benzene are at a substantially higher risk of contracting leukemia than normal individuals (Moran *et al.*, 1999).

2. Activation of chemotherapeutic agents

The same QR1 activity that protects cells against the deleterious effects of quinones such as menadione, reduces other quinones to metabolites that are more toxic than the parent compound (bioreductive activation). Since QR1 levels are elevated in some tumors, it has become a target of choice for the development of cancer chemotherapeutic prodrugs (Dehn *et al.*, 2005, 2006; Fourie *et al.*, 2002; Guo *et al.*, 2006; Nishiyama *et al.*, 1997; Okamura *et al.*, 2000; Reigan *et al.*, 2007; Suzuki *et al.*, 1999). Tumors and cancer cell lines in which increased QR1 expression has been observed include those from the lung, liver, colon, breast, and brain. Detailed studies suggest that breast cancer and non-small cell lung carcinoma (NSCLC) may be especially relevant targets for antitumor compounds that are efficiently activated by QR1 (Cummings *et al.*, 2003; Danson *et al.*, 2004; Kolesar *et al.*, 2002).

QR1 *enhances* the cytotoxicity and mutagenicity of a wide variety of compounds (De Flora and Ramel, 1988). For example, it reduces one of the nitro groups of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) to its 4-hydroxyl-amino metabolite, generating a highly cytotoxic bifunctional antitumor alkylating agent (Knox *et al.*, 1988). A similar nitroreductase activity may be important in the bioactivation of nitroquinoline-*N*-oxide (Sugimura *et al.*, 1966). Studies in many tumor cell lines have associated elevated QR1 activity with increased response to mitomycin C, anthracyclines,

and aziridinyI-benzoquinones (Boland *et al.*, 1991; Ross *et al.*, 1993; Siegel *et al.*, 1990; Traver *et al.*, 1992). For example, cell lines with increased QR activity are more sensitive to mitomycin C (Begleiter *et al.*, 1988, 1989) and this sensitivity is reversed by dicoumarol, a QR1 inhibitor. A cell line susceptible to the development of cancer had low levels of QR and was mitomycin C resistant (Marshall *et al.*, 1991). More importantly, an association between the resistance to mitomycin C and a decrease in QR1 activity was reported for mitomycin-resistant cells of human colon carcinoma (Pan *et al.*, 1995). Reduction of these antitumor compounds gives rise to reactive intermediates that can undergo nucleophilic additions to DNA (N7 of adenine) and other macromolecules, suggesting a possible mechanism for their cytotoxicity.

In the last few years, there has been an explosion in the number of groups developing compounds that can be activated by QR1 (Craig *et al.*, 1999; Flader *et al.*, 2000; Hargreaves *et al.*, 2000; Knox *et al.*, 2000; Loadman *et al.*, 2000; Miskiniene *et al.*, 1999; Okamura *et al.*, 2000; Phillips, 1999; Schulz and Skibo, 2000; Xing and Skibo, 2000). Some of these studies have used the structures of the complexes to guide design. Active site differences between the rat structure and the human QR1 (hQR1) suggest better avenues for design improvement. In addition, the structures of hQR1 in complex with three chemotherapeutic benzo- and indolequinones have suggested empirical rules that correlate QR1 reactivity of these drugs with the nature of the substituents (see below) (Faig *et al.*, 2001).

3. Role in the biosynthesis of blood coagulation proteins

QR is potently inhibited by dicoumarol and related vitamin K antagonists (Ernster *et al.*, 1960, 1962), and was originally isolated as a vitamin K reductase by Martius (1963). Based on this evidence, a role for QR in blood coagulation has been proposed. The hydroquinone of vitamin K is involved in the CO₂-dependent γ -carboxylation of glutamate residues of several coagulation proteins. Oxidized vitamin K (Suttie, 1985) requires reduction to the hydroquinone for the cycle to continue. QR is at least one of the mechanisms for regenerating the reduced form of vitamin K, and has been implicated directly in this process (Wallin and Hutson, 1982; Wallin *et al.*, 1978).

B. Mechanism of protective function of quinone reductase

The toxicity of quinones, ubiquitous dietary components, stems from: (a) the ability of quinones to act as Michael reaction acceptors and to undergo alkylation reactions, resulting, for instance, in the depletion of glutathione levels; and (b) the one-electron reduction of quinones (e.g., by NADPH:cytochrome P-450 reductase) that leads to the generation of semiquinones which readily react with molecular oxygen to generate superoxide and other reactive oxygen species. The resultant oxidative stress

is counteracted by the obligatory two-electron reductions promoted by QR1 (Iyanagi, 1987; Iyanagi and Yamazaki, 1970). The importance of the balance between one- and two-electron quinone reducing systems is shown in Fig. 4.1.

Several alternative mechanisms have been proposed for two-electron reductions (Brunmark and Cadenas, 1989). However, there is strong evidence suggesting that the two halves of the reaction catalyzed by QR1 are both hydride transfers (see below).

C. Structure of QR1

1. Overall structure

The structures of the rat (rQR1), mouse (mQR1), and human (hQR1) QR1 enzymes, as well as the structure of the human homolog QR2 have been reported, alone and in complex with substrates, products, inhibitors, and chemotherapeutic compounds. (For the general description of QR1, we will use the structure of the rat enzyme, but the others are quite similar.)

QR1 is a highly intertwined homodimer. Each subunit (monomer of 273 amino acids) contains two separate domains: a catalytic domain (residues 1–220) folded in a predominantly α/β structure, and a small C-terminal domain (residues 221–273) formed by an antiparallel hairpin followed by one helix and several loops (Fig. 4.2A and B). The overall fold of the catalytic domain

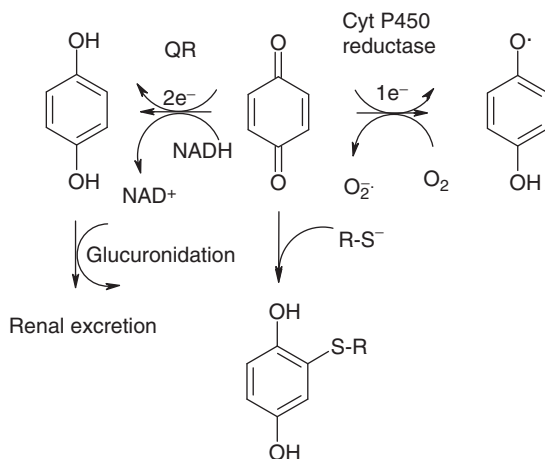


Figure 4.1 General reactions of dietary quinones. There are two deleterious reactions: (1) a one-electron reduction of a quinone by cytochrome 450 reductase produces a semiquinone that reacts readily with molecular oxygen to regenerate the quinone and produce radical superoxide; (2) reduced thiol compounds such as glutathione can react with quinones via a Michael addition with complete loss of the thiol compound. QR1 carries out an obligatory two-electron reaction of the quinones that results in a hydroquinone that through glucuronidation is targeted for renal excretion.

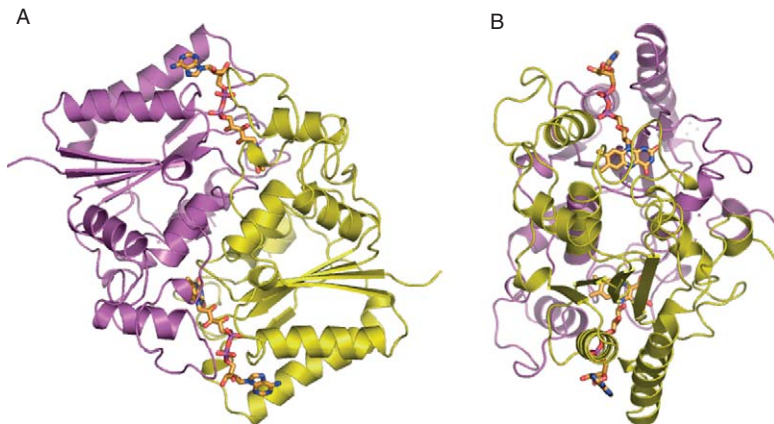


Figure 4.2 Overall structure of the QR1 dimer. (A) View of the dimer with the twofold axis perpendicular to the plane of the paper. The β -sheets of the two monomers (yellow and purple) are seen edge-on with the surrounding helices above and below the sheets. Each FAD is bound at an equivalent interface between the two monomers. (B) View of the dimer with the twofold axis running horizontally in the plane of the paper. This view shows that all portions of the structure of the FAD make contact with residues of the two monomers along the interface.

resembles that of other flavoproteins: a twisted central parallel β -sheet surrounded on both sides by connecting helices (Fig. 4.2A and B). The topology of the catalytic domain is not that found in other FAD containing proteins, but rather the topology of *Clostridium* flavodoxin (Smith *et al.*, 1977), an FMN containing protein. Alignment of the structures of QR1 and *Clostridium* flavodoxin shows that 80 α carbons can be aligned with an rms deviation between the two molecules of 1.8 Å even though they show very little sequence homology. Within this portion of the structure, QR1 has an additional β - α - β motif—43 residues long—between strand 2 and helix 2, instead of the direct connection found in flavodoxin (Smith *et al.*, 1977).

2. FAD binding site

The isoalloxazine moiety of FAD interacts with residues in several loops at one end of the dimer (L1 and L4 of one monomer, and L3 and L5 of the other monomer). Residues Tyr¹⁰⁴, Trp¹⁰⁵, Phe¹⁰⁶, and the main chain of residue Leu¹⁰³ interact directly with the rings and appear to anchor the isoalloxazine moiety. The two oxygens of the ring (O2 and O4) form hydrogen bonds with main chain NH groups of the protein, O4 with Phe¹⁰⁶, and O2 with Gly¹⁵⁰. The ring nitrogens also form hydrogen bonds with NH groups of the protein—N1 with Gly¹⁴⁹, N5 with Trp¹⁰⁵. Two residues from one monomer—Tyr¹⁰⁴ and Trp¹⁰⁵—and four residues from the other monomer—Ile⁵⁰, Tyr⁶⁷, Pro⁶⁸, and the main chain of Glu¹¹⁷—form a pocket for the two methyl groups of the isoalloxazine. The ribitol

interacts with the side chains of residues from the loop that connects strand 3 and helix 3. O2 of flavin is hydrogen bonded to the main chain carbonyl of Leu¹⁰³. The diphosphate of FAD binds at the amino-terminal end of helix 1 and in close proximity to the loop connecting strand 1 to helix 1. The two phosphates make several specific contacts with groups in the protein: hydrogen bonds to the main chain NH of Asp¹⁸, to the N of His¹¹, to a water molecule that is hydrogen bonded to the OH of Tyr¹⁰⁴, and to the N ϵ of Gln⁶⁶. The ribose is bound by residues from helix 1 and by the loop connecting strand 1 to helix 1. The adenine ring lies along helix 5 and interacts most strongly with Arg²⁰⁰. N3 makes a hydrogen bond with the guanidinium NH1 and the ring makes contacts with the main chain and with CH₂ groups of the side chain. It also interacts with the main chain of residues Thr¹⁵, Ser¹⁶, and Phe¹⁷ and with the side chains of Ala²⁰ and Leu²⁰⁴.

3. NADP⁺ binding site

The nicotinamide of NADP⁺ and ring C of the FAD isoalloxazine participate in a stacking interaction. The average distance between the planes of the two rings is ~ 3.4 Å. The binding site for NADP⁺ involves not only residues of the same subunit that binds FAD, but also residues from the other subunit of the dimer. The oxygen atom of the carboxamide (O7) makes two hydrogen bonds: one with the OH of Tyr¹²⁶, the other with the OH of Tyr¹²⁸ of the second monomer. The side chain of Phe¹⁷⁸, also of the second monomer, stacks against the nicotinamide ring. The O2' of the nicotinamide ribose makes a hydrogen bond with the N ϵ of His¹⁶¹ and the C3' is in van der Waals contact with the S of Met¹⁵⁴. The O3' appears to interact with the center of the aromatic ring of Tyr¹²⁸. The AMP moiety interacts mainly with the hairpin loop formed by strands 8 and 9 of the other monomer: the ribose makes contacts with Phe²³² and Phe²³⁶, and the adenine moiety with the main chain of residues in the loop. The main chain NH of Phe²³² forms a hydrogen bond with one of the oxygens of the phosphate at the O2' position of the sugar. This hydrogen bond is not present when NADH is the cofactor and can explain the difference in affinities between NADH and NADPH. The sequence TTGGSGS (147–153), which has been suggested to be part of the NADH binding site (Liu *et al.*, 1989; Ma *et al.*, 1992; Prochaska, 1988), does not make any specific interactions with the dinucleotide, but it appears that the main chain of these residues is packed very closely with the sugar phosphates of both cofactors: Gly¹⁴⁹ interacts with the ribitol phosphate of FAD and Gly¹⁵⁰ with the ribose oxygen O4' of the nicotinamide and with O2 of the flavin. As in other nucleotide binding proteins containing this consensus sequence, the two glycines appear to be necessary to allow close approach between the main chain and the cofactor.

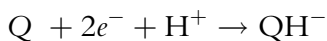
4. Substrate binding: Duroquinone

Duroquinone (2,3,5,6-tetramethylbenzoquinone, a QR1 substrate) occupies a position very similar to that of the nicotinamide ring of NADP⁺ when it is bound to the enzyme. It stacks with the isoalloxazine ring (the distance between the two rings is 3.4 Å) and one of its oxygens forms hydrogen bonds with the OH groups of Tyr¹²⁶ and Tyr¹²⁸ (Fig. 4.3).

D. Mechanisms of electron transfer from NADH to quinones

1. Solution studies

Studies of nonenzymatic quinone reduction *in solution* mediated by the NAD analog NADA provide insight into the mechanism of enzymatically mediated hydride transfer. Carlson and Miller (1985) demonstrated that (a) the rate of spontaneous reduction of quinones by NADH is first order with respect to both reactants, and (b) significant primary kinetic isotope effects are observed with deuterated NADH, NADH-d₁ and NADH-d₂. Furthermore, the log of the rate constants for the reduction of a large number of quinones is correlated ($r = 0.98$) with the redox potential of the following reaction:



These results argue for direct hydride transfer from NADH to the quinone. It is interesting that the pK_a of the product hydroquinone has a strong effect on the rate: those with low pK_a s were much more reactive than those with high pK_a values. These and other observations argue that the product of the

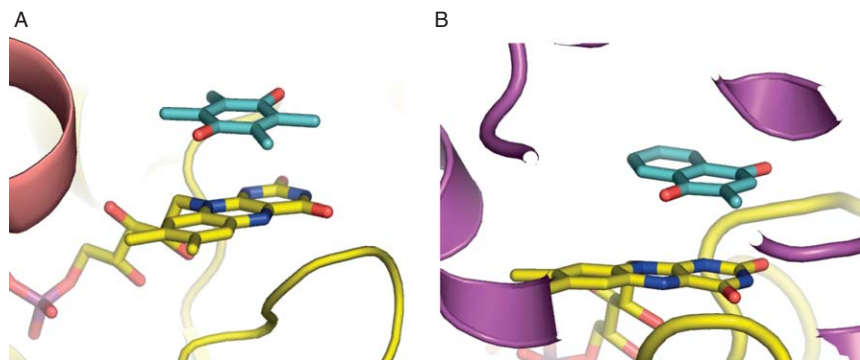


Figure 4.3 Binding of substrates to QR1 and QR2. (A) Binding of duroquinone (2,3,4,6-benzoquinone) to QR1. The main chains of the two monomers contributing to the binding are shown in different colors. The substrate is clearly stacked with the isoalloxazine ring of the FAD. (B) Menadione (Vit K3) bound to QR2. Binding is highly similar to that of panel (A) suggesting that QR1 and QR2 use the same mechanism.

rate limiting step is the phenolate hydroquinone, because the hydroquinone products that can best tolerate loss of a proton are the most reactive. The identity of the quinone acceptor atom is still not known. The C2 carbon can be ruled out by the lack of labeling of the quinone with NADH-d₂. Although neither of the other two positions has been ruled out, [Carlson and Miller \(1985\)](#) favor the quinolic oxygen, because attack at the carbonyl carbon would result in the formation of an unstable intermediate.

2. QR1 catalyzed reaction

Since the nonenzymatic reduction of quinone to hydroquinone by reduced nicotinamide appears to be a direct hydride transfer ([Carlson and Miller, 1985](#); [Cavelier and Amzel, 2001](#)), it is puzzling why this enzyme requires a flavin cofactor for catalytic activity ([Prochaska and Talalay, 1986](#)). In contrast to other flavoproteins capable of reducing quinones, QR does not show a detectable e.p.r. signal when incubated in the presence of *p*-benzoquinone and NAD(P)H, indicating that in QR semiquinones are not released into the solution ([Iyanagi, 1987](#); [Iyanagi and Yamazaki, 1970](#)). Furthermore, upon reduction of the QR FAD by NADPH under anaerobic conditions ([Huang *et al.*, 1987](#)), the FAD absorbances at 370 and 450 nm disappear without the appearance of a red-shifted absorbance in the visible spectrum ([Huang *et al.*, 1987](#); [Prochaska, 1988](#)). The absence of increased absorbance beyond 500 nm after FAD reduction argues against the presence of a stable semiquinone form of FAD, and also against the existence of an FAD-disulfide redox relay system like that observed in glutathione reductase and related enzymes ([Pai and Schulz, 1983](#)).

The nicotinamide of NADH and the quinone substrate alternate binding to the same site in the enzyme ([Li *et al.*, 1995](#)). Bound in this site, both the nicotinamide and the quinone are ideally positioned for hydride exchange with the flavin. The structure of the rat enzyme and those of its complexes suggest that the enzymatic reaction occurs via a double hydride transfer—from NAD(P)H to flavin, and from flavin to quinone ([Li *et al.*, 1995](#)).

Based on the structures of two complexes—rQR1/NADP⁺ and rQR1/duroquinone (DQ)—it was proposed that in the QR1 catalyzed reaction, hydride is transferred in a manner analogous to the mechanism of spontaneous transfer in solution ([Fig. 4.4](#)). The structures of apo hQR1 and hQR1/DQ, determined to 2.0 Å resolutions, provided additional details about the enzymatic mechanism.

3. Bound conformations and mechanism

The position of the nicotinamide in QR1 is ideal for a direct hydride transfer from NADH to FAD⁺ in the first half of the reaction cycle: C4 of the nicotinamide is 4.02 Å from the flavin N5. After the hydride transfer, the FADH[−] has to become protonated. As mentioned, the N1 is hydrogen bonded to the NH of Gly¹⁴⁹, and there are no groups that can

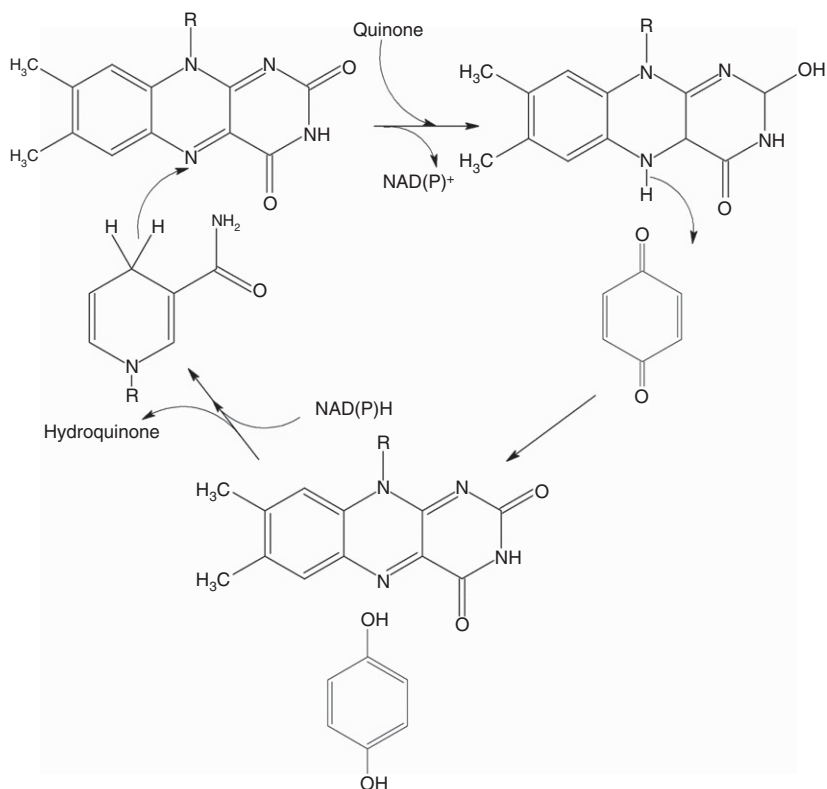


Figure 4.4 General ping-pong mechanism of quinone reduction by QR1. FAD is reduced NADH or NADPH by a direct hydride transfer. After NAD(P)⁺ leaves the enzyme, the quinone substrate binds and gets reduced by a hydride transfer from the reduced FADH₂. Note that in the reduced state, the FAD is in its enolic form, protonated O2.

donate a proton to compensate a charge if it develops in N1 (as commonly proposed in other systems). Thus, in QR1, the most likely tautomer is the enolic form with the negative charge in O2. Because O2 is already the acceptor atom of a hydrogen bond with Tyr¹⁵⁵, this tautomer can receive the proton from the OH of Tyr¹⁵⁵, which can, in turn, be stabilized by the positive charge of (or the transfer of a proton from) His¹⁶¹. The imidazole of His¹⁶¹ is close to the nicotinamide, so the net effect of this step (in addition to the transfer of the hydride from the nicotinamide to the isoalloxazine) is the movement of a positive charge over a very short distance from the imidazole ring of His¹⁶¹ to the nicotinamide. This process is reversed when the hydride is transferred to the quinone.

Binding of quinone substrate cannot occur until NAD(P)⁺ is released because, as mentioned above, the quinone and the nicotinamide share the

same binding site. This provides a simple rationale for the ping-pong mechanism of the enzyme (Li *et al.*, 1995). After nucleotide release, the quinone binds to the site freed by the nicotinamide in an orientation ideally suited to accept a hydride from the reduced FADH₂. The quinone is reduced by the hydride to hydroquinolate, and the isoalloxazine is oxidized to the quinone form. The proton on O2 is transferred back to the OH of Tyr¹⁵⁵. The imidazole ring of His¹⁶¹ becomes fully protonated again and can either transfer a proton to the hydroquinolate or simply stabilize its negative charge. The result of this second half of the reaction is, in addition to the hydride transfer, the transfer of a proton from the O2 of the flavin (accepted in the first half of the reaction) to the hydroquinolate.

The mechanism suggested by the structure provides a direct explanation of why the reaction catalyzed by QR is an obligatory two-electron reduction, that is, why there are no one-electron intermediates: *both* halves of the reaction involve hydride transfer—first from NADH to FAD and then from FADH₂ to the quinone. The charge relay formed by Tyr¹⁵⁵ and His¹⁶¹ system allows the reaction to take place without unfavorable charge separations.

E. Structural studies of chemotherapeutic prodrugs

As mentioned above, the same catalytic activity that protects cells from the deleterious effects of quinones and other compounds can carry out the reductive active activation of reactive quinones. The structures of the complexes of hQR1 with several prodrugs provided important information about the mode of binding of individual compounds with major implication with respect to the design of more effective prodrugs.

The structures of the complexes of human QR1 with several chemotherapeutic prodrugs have been reported. They include three indole-diones (ES936, ES1951, and ES1340), two azyridinyl-indole-diones (ARH019 and E09), and a diazyridinyl-benzoquinone (RH1) (Faig *et al.*, 2001; Winski *et al.*, 2001). The chemical structures of these compounds (Fig. 4.5 show that the indolequinones differ mainly in the nature of the substituents in position 2, 3, and 5 (Figs. 4.6 and 4.7). However, simple analysis of kinetic activity ($V_{\max}/K_M$) versus chemical formula showed very little correlation. A more compelling picture was obtained by the analysis of the three-dimensional structure of the complexes of human QR1 with these compounds. In addition to the details of the structures, important observations emerge from these studies. First, the structures show that the substrate binding site of the enzyme can modify its shape to accommodate diverse substrates. The most significant changes when accommodating to different substrates are large rotations of the side chain

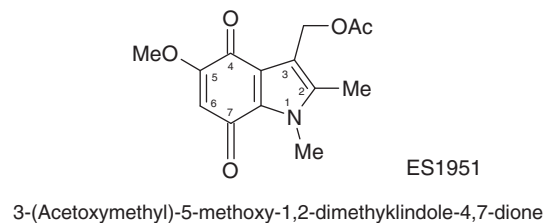
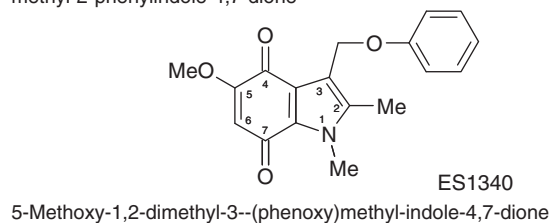
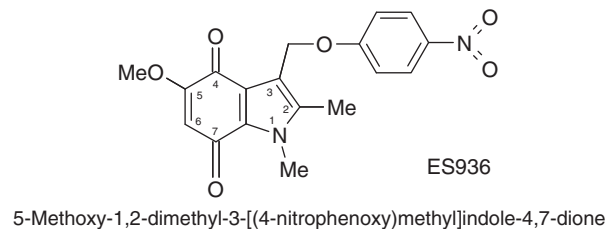
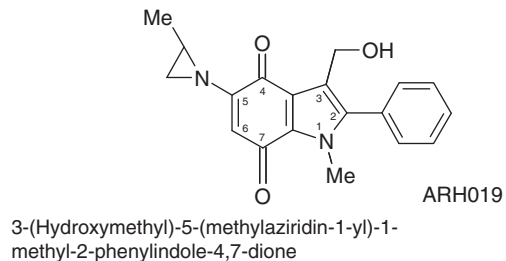
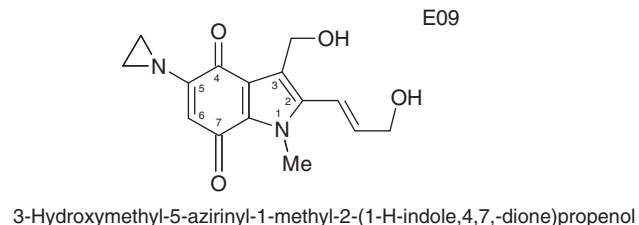
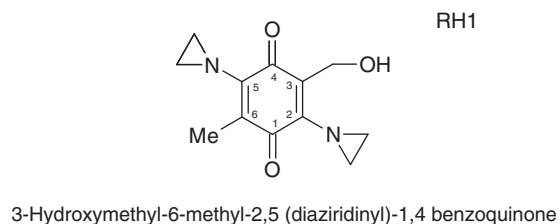


Figure 4.5 Chemical structure of the chemotherapeutic prodrugs targeted to QR1. The list includes five indolequinones and one benzoquinone. Three of the compounds contain aziridinyl substituents.

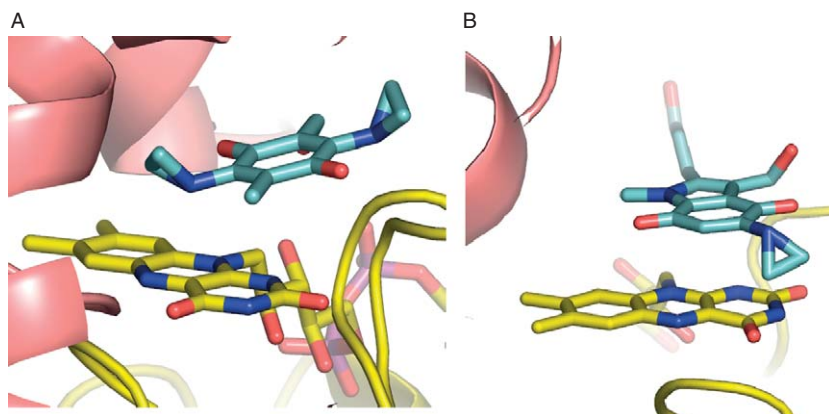


Figure 4.6 Two chemotherapeutic compounds bound to QR1. (A) RH1 (2,5-aziridinyl-3-hydroxy-6-methyl-1,4-benzoquinone) bound to QR1. (B) E09 bound to QR1.

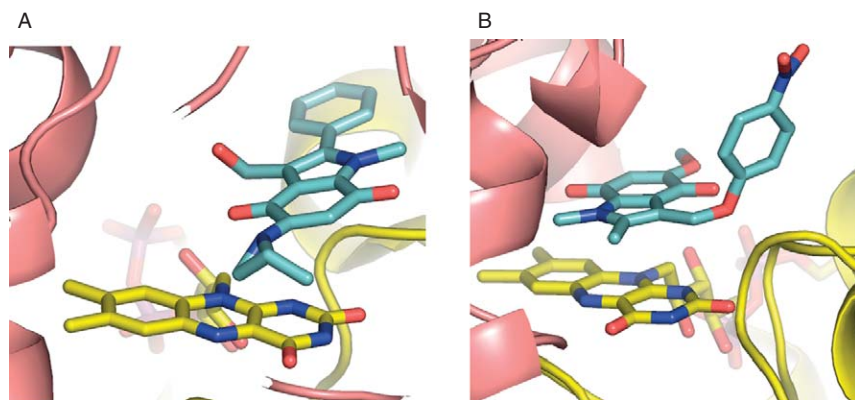


Figure 4.7 Two chemotherapeutic compounds bound to QR1. (A) ARH019 bound to QR1. (B) ES936 bound to QR1. The nitrophenol is an excellent leaving group making this compound particularly reactive.

of Tyr¹²⁸ around χ_1 and χ_2 , but other small changes also take place. Second, quite similar compounds can bind to QR1 in different orientations. For example, the two indolequinones, E09 and ARH019, bind to the enzyme in different orientations, while RH1, a benzoquinone, and ARH019, an indolequinone, bind in similar orientations. The structures show that mode of binding correlates with the size of the substituents at positions 2 and 3 of the indole. The main conclusions of these studies are:

- The active site of QR1 is highly plastic and changes to accommodate ligands of different sizes and shapes.
- Homologous drugs may bind in different orientations because QR1 recognizes ligands mostly by hydrophobic interactions with only few H-bonds.
- The different modes of binding correlate with the kinetic behavior of drugs with different substituents and of a given drug with QR1 of different animal species.
- The observed binding modes of indolequinones with different substituents at positions 2 and 3 of the indole provide a rationale for the design of more effective indolequinone QR1-activated prodrugs.

ES936, one of the indolequinone prodrugs, is an irreversible inhibitor of QR1. The mechanism of inhibition was studied by a combination of X-ray diffraction and mass spectrometry (Winski *et al.*, 2001) and it was found that ES936 is a mechanism-based inhibitor in which the compound is activated in a manner that probably reflects the activation mechanism in its role as a chemotherapeutic prodrug.

III. QUINONE REDUCTASE TYPE 2

For a comprehensive review on QR2 see Vella *et al.* (2005). In contrast to QR1, which is ubiquitously expressed, QR2 is mainly expressed in several mammalian tissues, including heart, liver, skeletal muscle, and kidney (AbuKhader *et al.*, 2005). Like QR1, QR2 is a cytosolic enzyme active as a homodimer, with one FAD cofactor bound per subunit. QR2 is smaller than QR1, lacking the 47 C-terminal amino acid residues. The protein sequences of human QR1 and QR2 can be aligned without insertions or deletions, and are 49% identical over their shared length.

Although QR2 is expressed in many of the same tissues as QR1 (AbuKhader *et al.*, 2005), it does not appear to be induced in conjunction with QR1 (Begleiter and Fourie, 2004). In addition, while QR1 reduces quinones using reducing equivalents from NADH or NADPH, QR2 is unable to use these conventional phosphorylated nicotinamides as reductants, and has been shown to react only with a specific subset of nonphosphorylated nicotinamide derivatives, including *N*-methyl, *N*-ribosyl-, and *N*-phenyl-dihydronicotinamide (Amzel *et al.*, 1986; Bayney *et al.*, 1987; Begleiter and Fourie, 2004). Furthermore, QR2 is only marginally inhibited by characteristic QR1 inhibitors, but is potently inhibited by polycyclic aromatic hydrocarbons (Begleiter and Fourie, 2004; Buryanovskyy *et al.*, 2004). These and other factors have made it difficult to identify the physiological function of QR2. Activities that have been proposed to be important

physiological functions of QR2 include being the physiological target of antimalarial drugs such as chloroquin, and of resveratrol (Buryanovsky *et al.*, 2004), the compound present in red wine that is thought to have chemoprotective and antiaging properties. QR2 has been also shown to be identical to a protein previously identified as the melatonin binding site MT₃ and was suggested to be involved in increasing the toxicity of menadione and other quinones *in vivo* (Vella *et al.*, 2005).

A. Structure of human QR2

The structures of QR2 and of several of its complexes have been determined. The structure of QR2 resembles that of QR1 but without its C-terminal domain that is almost completely missing in QR2. Surprisingly, the initial structure determination (Foster *et al.*, 1999) showed that the C-terminus of QR2 contains a Zn²⁺ ion coordinated by two histidine nitrogens, one cysteine thiol, and a main chain carbonyl group. The same Zn²⁺ ion was observed in the other QR2 structures. The structure of the complex of QR2 with Vit K3 (menadione, Fig. 4.3) (Foster *et al.*, 1999) shows that the vitamin binds with its quinone oxygens in positions highly similar to those occupied by the simpler quinones (such as duroquinone, Fig. 4.3), suggesting that Vit K3 can be reduced by either QR1 or QR2 via the same mechanism used by the enzyme to reduce all other quinones.

The prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is activated by QR2 3,000 times more effectively (in terms of $k_{\text{cat}}/K_{\text{M}}$) than by QR1. Several structures of the complex of QR2 with the chemotherapeutic prodrug

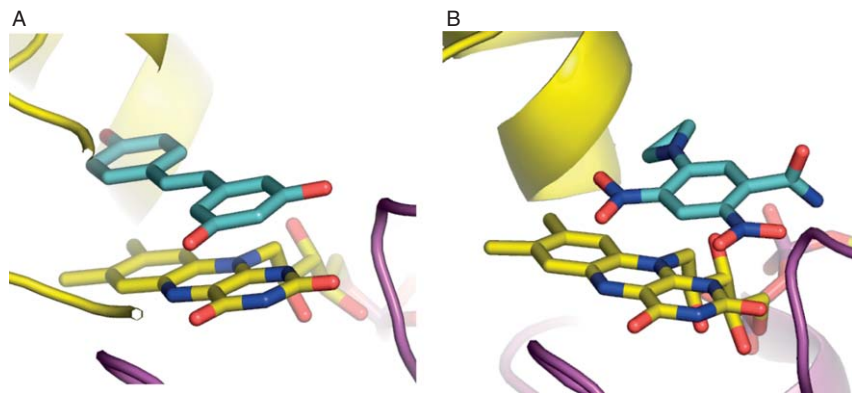


Figure 4.8 Two compounds bound to QR2. (A) Resveratrol. Resveratrol, a potent inhibitor of QR2, has been associated with the beneficial health effects of red wine. The compound binds QR2 with high affinity (35 nM) by making several hydrogen bonds (not shown). (B) CB1954. This compound, after activation by QR2, becomes highly cytotoxic.

CB1954 have been reported (Fig. 4.8) (AbuKhader *et al.*, 2005; Buryanovskyy *et al.*, 2004). The position and orientation of CB1954 bound to QR2 is very similar to that of bound quinones, with one of the nitro groups and the carboxamide occupying the positions of the quinolic oxygens. One of the nitro groups forms hydrogen bonds with the side chain of Asn¹⁶¹ of QR2 in a way that orients the other nitro group for the hydride transfer from the FADH₂. Residue 161, an Asn in QR2, is a His in QR1, which may explain why CB1854 is not a good substrate of QR1. In agreement with this prediction, mutation of Asn¹⁶¹ in QR2 to histidine, resulted in loss of enzymatic activation of CB1954 without affecting the rate of reduction of menadione (Fu *et al.*, 2005).

Resveratrol, a compound associated with the health effects of red wine, has been shown to be a potent inhibitor of QR2 ($K_i = 35$ nM). In the structure of the complex of QR2 with resveratrol, all three hydroxyl groups of the inhibitor form hydrogen bonds with amino acids from the enzyme, placing the flat ring system of resveratrol and the isoalloxazine ring of FAD in a parallel orientation (Fig. 4.8). This mode of binding suggests that other polyphenols, such as flavonoids, may also be inhibitors of QR2 (Buryanovskyy *et al.*, 2004).

IV. DISCLAIMER

This chapter deals with structural, functional, and mechanistic aspects of quinone reductases with emphasis on information obtained from structural studies. Many interesting aspects of this field were left out because they were already thoroughly reviewed or because they deserve full reviews that will be outside the scope of this volume. These areas include the mechanism of induction of QR1 as part of the phase 2 detoxification cascade under the control of the antioxidant responsive elements (AREs) (Begleiter and Fourie 2004; Dinkova-Kostova and Talalay 2000; Fahey and Talalay 1999; Itoh *et al.*, 2004; Jaiswal, 2004), the effects of inducers of this response such as sulforaphane and related compounds (Fahey and Talalay, 1999), the effects of QR1 mutations in humans and in animals models, and the multiple possible functions of QR2 (Chen *et al.*, 2000; Vella *et al.*, 2005).

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QUINONE OXIDOREDUCTASES AND VITAMIN K METABOLISM

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Abstract

Vitamin K₁, K₂, and K₃ are essential nutrients associated with blood clotting and bone metabolism. Quinone oxidoreductases [NAD(P)H:quinone oxidoreductase 1 (NQO₁) and NRH:quinone oxidoreductase 2 (NQO₂)] are among the selected enzymes that catalyze reduction of vitamin K to vitamin K hydroquinone. NQO₁ catalyzes high affinity reduction of vitamin K₃ but has only weak affinity for reduction of vitamin K₁ and K₂. Vitamin K hydroquinone serves as a cofactor for vitamin K γ -carboxylase that catalyzes γ -carboxylation of specific glutamic acid residues in Gla-factors/proteins leading to their activation and participation in blood clotting and bone metabolism. Concomitant with Gla modification, a reduced vitamin K molecule is converted to vitamin K epoxide, which is converted back to vitamin K by the enzyme vitamin K epoxide reductase to complete vitamin K cycle. Vitamin K is also redox cycled. One-electron reduction of vitamin K₃ leads to the formation of semiquinone that in the presence of oxygen is oxidized back to vitamin K₃. Oxygen is reduced to generate reactive oxygen species (ROS) that causes oxidative stress and cytotoxicity. Vitamin K is used as radiation sensitizer or in mixtures with other chemotherapeutic drugs to treat several types of cancer. ROS generated in

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redox cycling contributes to anticancer activity of vitamin K. NQO1 competes with enzymes that redox cycle vitamin K and catalyzes two-electron reduction of vitamin K₃ to hydroquinone. This skips formation of semiquinone and ROS. Therefore, NQO1 metabolically detoxifies vitamin K₃ and protects cells against oxidative stress and other adverse effects. On the contrary, NQO2 catalyzes metabolic activation of vitamin K₃ leading to cytotoxicity. The role of NQO1 and NQO2 in metabolic detoxification and/or activation of vitamin K₁ and K₂ remains to be determined. Future studies are also required to identify the enzymes that catalyze high affinity reduction of vitamin K₁ and K₂ to hydroquinone for use in γ -carboxylation reactions.

Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase 1; NQO2, NRH:quinone oxidoreductase 2; NRH, dihydronicotinamide riboside; VKOR, vitamin K epoxide reductase; GGCX, γ -glutamyl carboxylase; Vitamin K₁, phyloquinone; Vitamin K₂, menaquinones; Vitamin K₃, menadione. © 2008 Elsevier Inc.

I. VITAMIN K AND VITAMIN K CYCLE

Vitamin K (“Koagulation” in German) denotes a group of lipophilic, and hydrophobic, vitamins that are chemical derivatives of 2-methyl-1,4-naphthoquinone (Fig. 5.1). Vitamin K₁ (phyloquinone) invariably contains

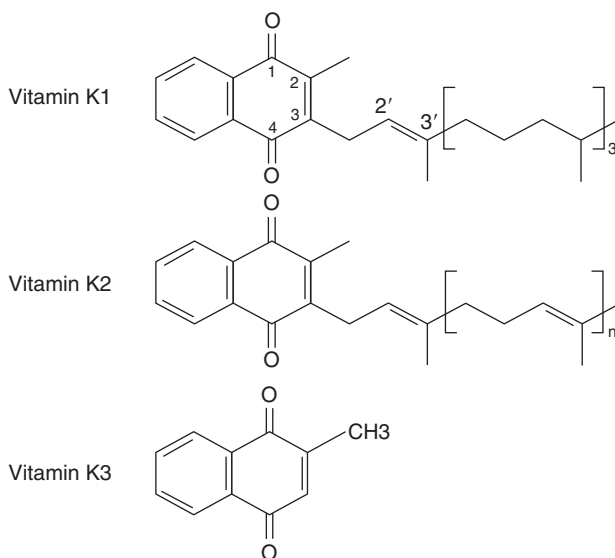


Figure 5.1 Chemical structures of K vitamins. Phylloquinone is vitamin K₁, menaquinones are vitamin K₂, and menadione is vitamin K₃.

in its side chain four isoprenoid residues, one of which is unsaturated. Plants synthesize vitamin K1. Green leafy vegetables such as kale, spinach, and broccoli are rich sources of vitamin K1 (Shearer, 1995; Stafford, 2005). Vitamin K2 (menaquinones) has side chains composed of a variable number of unsaturated isoprenoid residues; generally they are designated as MK- n , where n specifies the number of isoprenoids (Shearer, 1995). Bacteria synthesize a range of vitamin K2 using repeating 5-carbon units in the side chain of the molecule (Shearer, 1995). Vitamin K2 is found in liver, milk, cheese, and fermented soy products. Intestinal bacteria also produce vitamin K2 that is absorbed. Both vitamin K1 and K2 are naturally occurring vitamins. Vitamin K3 or menadione is a chemically synthesized derivative used for animal feeding (Shearer, 1995; Stafford, 2005). It is noteworthy that vitamin K is not stored in the body. Therefore, vitamin K has to come from nutrients and/or intestinal bacteria. As discussed later in this review, vitamin K is also recycled and reused.

Vitamin K is involved in the carboxylation of selected glutamate residues in proteins to form γ -carboxyglutamate residues (abbreviated Gla-residues) (Fig. 5.2). Gla-residues bind to calcium and are essential for the biological activity of all known Gla-proteins (Shearer, 1995; Stafford, 2005). Fourteen human Gla-proteins have been identified so far. These proteins regulate three physiological processes that include blood clotting, bone metabolism, and vascular biology (Shearer, 1995; Stafford, 2005). Gla-proteins [prothrombin (factor II), factors VII, IX, X, protein C, protein S, and protein Z] participate in blood clotting. Bone Gla-protein or osteocalcin and matrix Gla-protein (MGP) participate in bone metabolism. The functions of some of the Gla-proteins remain unknown.

Vitamin K-dependent γ -glutamyl carboxylase (GGCX) catalyzes Gla modification of proteins (Presnell and Stafford, 2002; Suttie, 1985). This enzyme is a part of the vitamin K cycle and requires vitamin K hydroquinone to catalyze Gla modification of proteins (Fig. 5.2). Concomitant with Gla modification, a reduced vitamin K molecule is converted to vitamin K epoxide. The vitamin K epoxide is converted back to vitamin K by the enzyme vitamin K epoxide reductase (VKOR). The conversion of vitamin K to vitamin K hydroquinone is catalyzed by NAD(P)H:quinone oxidoreductase 1 (NQO1), previously known as DT diaphorase or vitamin K epoxide reductase or others as yet unknown enzymes (Stafford, 2005).

II. QUINONE OXIDOREDUCTASES

Quinone oxidoreductases [NAD(P)H: quinone oxidoreductase 1 NQO1, and NRH: quinone oxidoreductase 2 (NQO2)] are cytosolic proteins that catalyze metabolic reduction of quinones and its derivatives

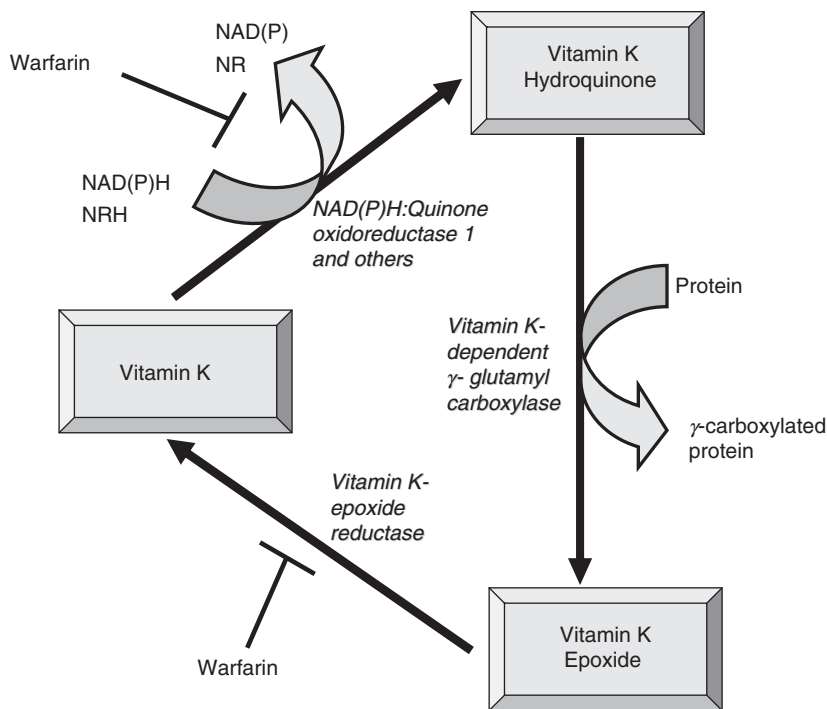


Figure 5.2 Vitamin K cycle. Vitamin K is reduced to vitamin K hydroquinone by NAD(P)H:quinone oxidoreductase 1 (NQO1). Vitamin K-dependent carboxylase utilizes vitamin K hydroquinone to carboxylate glutamate residues in proteins to form γ -carboxylglutamate. During this process, the vitamin K hydroquinone is converted to vitamin K epoxide. Vitamin K epoxide reductase converts vitamin K epoxide to vitamin K.

(Long and Jaiswal, 2000; Radjendirane *et al.*, 1997; Riley and Workman, 1992; Ross *et al.*, 2000; Talalay *et al.*, 1995). The cytosolic NQO1 activities, purified from rat liver and human adipose tissue, have been characterized and cloned (Bayney *et al.*, 1989; Jaiswal, 1991; Jaiswal *et al.*, 1988; Lind *et al.*, 1982; Robertson *et al.*, 1986; Williams *et al.*, 1986). The dicoumarol-sensitive NQO1 is a cytosolic protein of 274 amino acid residues (MW = 30,880). It has been shown that the two-electron reduction of quinones, catalyzed by NQO1, competes with the one-electron reduction, catalyzed by cytochromes P450 and P450 reductase (Joseph and Jaiswal, 1994). This produces a comparatively stable hydroquinone that is removed by conjugation with glutathione, UDP-glucuronic acid, etc. (Lind *et al.*, 1982; Radjendirane *et al.*, 1997; Riley and Workman, 1992; Ross *et al.*, 2000; Talalay *et al.*, 1995). The two-electron reduction of quinones does not result in the formation of free radicals (semiquinones) and highly reactive oxygen species (ROS), hence protecting cells against the adverse effects of

quinones and their derivatives. The NQO1 is generally accepted as protective against quinone toxicity. However, in many instances, NQO1 has been shown to activate quinones (Begleiter *et al.*, 1997; Boyer, 1997; Rauth *et al.*, 1997; Ross *et al.*, 1994; Sartorelli *et al.*, 1994; Workman, 1994; Workman and Stratford, 1993). The hydroquinones produced by NQO1 can auto-oxidize to generate ROS or directly alkylate DNA. In these instances, NQO1 catalyzes activation of such compounds to their ultimate toxic forms. This property of NQO1, along with the observation that NQO1 is expressed at higher levels in certain tumor types, has been used to develop bioreductive chemotherapeutic agents.

NQO2 is a second member of quinone oxidoreductase family of proteins. The cDNA and gene encoding NQO2 were isolated from human liver (Jaiswal, 1994; Jaiswal *et al.*, 1990). The human NQO2 gene encodes a protein of 231 amino acids (MW = 25,956). The human NQO2 carboxy-terminus is 43 amino acids shorter than the human, rat, and mouse NQO1. The cofactor requirement for protein activity is very selective, pointing out dihydronicotinamide riboside (NRH) for NQO2, and NAD(P)H for NQO1 as an electron donor (Wu *et al.*, 1997; Zhao *et al.*, 1997). Although NQO2 is resistant to typical activity inhibitors of NQO1, such as dicoumarol, cibacron blue, and phenindone, NQO2 is inhibited by flavones such as quercetin (Wu *et al.*, 1997). Benzo(a)pyrene is another known inhibitor of NQO2 (Zhao *et al.*, 1997). Even though overlapping substrate specificities have been observed for NQO1 and NQO2, such as for CB1954 activation, significant differences exist in relative affinities for the various substrates (Knox *et al.*, 2000; Wu *et al.*, 1997). Analysis of the crystal structure of NQO2 revealed that NQO2 contains a specific metal binding site, which is not present in NQO1 (Foster *et al.*, 1999). Cellular studies have shown a role of NQO2 in metabolic activation of CB1954 leading to cytotoxicity and cell death (Celli *et al.*, 2006; Knox *et al.*, 2000).

The physiological function of NQO1 and NQO2 proteins is unclear at present. Previous studies have suggested a role of NQO1 in oxidation of NAD(P)H and reduction of CoQ (Beyer *et al.*, 1996; Ernster, 1967; Turunen *et al.*, 2004). CoQ, like NQO1, is present in all cells and membranes and has multiple functions including electron carrier in the mitochondrial respiratory chain and regulation of physicochemical properties of membranes (Turunen *et al.*, 2004). CoQ reduction to CoQH₂ is required for antioxidant function of CoQ (Turunen *et al.*, 2004). It is suggested that NQO1 catalyzes this reduction of CoQ (Beyer *et al.*, 1996). The information on physiological role of NQO2 is lacking. The reports have identified NQO2 as melatonin binding site MT3 with unknown functions in CNS control of circadian rhythm (Nosjean *et al.*, 2000, 2001).

NQO1 and NQO2 activity is ubiquitously present in all types of tissues (Radjendirane *et al.*, 1997; Riley and Workman, 1992; Ross *et al.*, 2000; Talalay *et al.*, 1995). Several investigators have observed large variations in

NQO1 activity among different individuals, tissue types of the same individual, and between normal and tumor tissues (Radjendirane *et al.*, 1997; Riley and Workman, 1992; Ross *et al.*, 2000; Talalay *et al.*, 1995). Alterations in NQO1 and NQO2 genes in tumor tissues have been reported (Radjendirane *et al.*, 1997; Strassburg *et al.*, 2002). The NQO1 gene expression is induced in response to xenobiotics, antioxidants, oxidants, heavy metals, UV light, and ionizing radiation (Jaiswal, 2004; Radjendirane *et al.*, 1997). Interestingly, NQO1 is part of an electrophilic- and/or oxidative stress-induced cellular defense mechanism that includes the induction of more than two-dozen genes (Jaiswal, 2004; Radjendirane *et al.*, 1997). The coordinated induction of these genes, including NQO1, provides necessary protection for cells against free radical damage, oxidative stress, and neoplasia. Several studies have reported that many diverse chemicals block carcinogenesis (Wattenburg, 1983, 1985; Zhang *et al.*, 1992, 1994). The capacity of these chemicals to block carcinogenesis correlates with their capacity to induce NQO1 and other detoxifying enzymes. Zhang *et al.* followed induction of the NQO1 activity in mouse hepatoma (Hepa-1) cells and isolated a compound "sulforaphane" from Saga broccoli (Zhang *et al.*, 1992). Recently, sulforaphane has been shown to block the formation of mammary tumors in Sprague-Dawley rats treated with single dose of 9,10-dimethyl-1,2-benzanthracene (Zhang *et al.*, 1994). The expression of NQO2 gene is also activated in response to xenobiotics and antioxidants (Radjendirane and Jaiswal, 1999).

Human NQO1 gene has been localized to chromosome 16q22 (Jaiswal *et al.*, 1999). A cytosine to thymidine (C → T) polymorphism in exon 6 of human NQO1 gene produces a proline to serine (P187S) substitution that destabilizes and inactivates the enzyme (Siegel *et al.*, 2001; Traver *et al.*, 1992). The mutant NQO1 is rapidly degraded via ubiquitination and proteasome degradation (Siegel *et al.*, 2001). Individuals carrying both mutated genomic alleles are completely lacking in NQO1 activity, whereas individuals who are heterozygous with one mutated allele have low-to-intermediate NQO1 activity compared with wild-type individuals (Siegel *et al.*, 1999). Approximately 2–4% human individuals are homozygous and 20–25% are heterozygous for this mutation (Kelsey *et al.*, 1997; Rosvold *et al.*, 1995; Siegel *et al.*, 1999, 2001; Schulz *et al.*, 1998; Weincke *et al.*, 1997). Human NQO2 gene is precisely localized to chromosome 6p25 and its gene locus is highly polymorphic (Jaiswal *et al.*, 1999). However, the polymorphisms detected so far are in the intronic regions or nonsense mutations that did not lead to change in amino acids (Iida *et al.*, 2001; Jaiswal, 1991; Jaiswal *et al.*, 1999). A recent report has identified mutation in the first intron of NQO2 gene associated with decreased expression of NQO2 gene and clozapine-induced agranulocytosis in clozapine-treated schizophrenic patients (Ostrousky *et al.*, 2003). A 29-bp promoter polymorphism associated

with differential expression of NQO2 gene is reported (Harada *et al.*, 2001; Wang and Jaiswal, 2004). Human NQO2 gene promoter without the 29-bp insertion expressing higher NQO2 protein was found associated with increased NQO2 gene expression and increased susceptibility to Parkinson's disease (Harada *et al.*, 2001; Ostrousky *et al.*, 2003; Wang and Jaiswal, 2004).

NQO1-null and NQO2-null mice were generated (Long *et al.*, 2002b; Radjendirane *et al.*, 1998). The mice deficient in NQO1 and NQO2 gene expression were born and developed normal indicating that NQO1 and NQO2 do not play a role in mouse development. Further studies on NQO1-null mice revealed altered intracellular redox status and altered metabolism of carbohydrates, fatty acids, and nucleotides, and reduced accumulation of abdominal fat with age (Gaikwad *et al.*, 2001). In addition, the studies demonstrated that loss of NQO1 gene expression in NQO1-null mice led to myelogenous hyperplasia of bone marrow (Long *et al.*, 2002a). NQO1-null mice also demonstrated benzene toxicity (Bauer *et al.*, 2003) and significantly increased sensitivity to skin carcinogenesis in response to benzo(a)pyrene (Long *et al.*, 2000) and dimethylbenzanthracene (Long *et al.*, 2001). Similar to NQO1-null mice, myeloid hyperplasia of bone marrow was detected in NQO2-null mice (Long *et al.*, 2002b). NQO2-null mice like NQO1-null mice also demonstrated increased susceptibility to skin carcinogenesis when exposed to benzo(a)pyrene and dimethylbenzanthracene (Iskander *et al.*, 2004).

III. QUINONE OXIDOREDUCTASES AND REDUCTION OF VITAMIN K TO HYDROQUINONE

Blood coagulation is a process that protects animals and humans from bleeding to death. Vitamin K-dependent γ -carboxylation of specific glutamic acid residues in seven clotting factors (factors II, VII, IX, X, Z, C, and S) plays a significant role in the coagulation cascade leading to blood clotting (Furie *et al.*, 1999; Suttie, 1996). The term, coagulation cascade, refers to a series of events that stops bleeding through clot formation. Vitamin K-dependent γ -carboxylation of specific glutamic acid residues in factors II (prothrombin), VII, IX, and X that make up the core of coagulation cascade makes possible for them to bind calcium, a requirement for blood clot formation. The γ -carboxylation of specific glutamic acid residues in other three proteins activates them to perform additional functions in blood coagulation. The carboxylation of Z-protein enhances the action of thrombin by promoting its association with phospholipids in cell membranes. Proteins C and S are anticoagulant proteins that provide control and balance in the coagulation cascade. This is because uncontrolled clotting might be as life

threatening as uncontrolled bleeding. Vitamin K-dependent coagulation factors are synthesized in liver. Therefore, vitamin K deficiency not only leads to bleeding problems but also severe liver disease (Senzolo *et al.*, 2006). Some people are at risk of forming blood clots, which could block the flow of blood in arteries of the heart, brain, or lungs, resulting in heart attack, stroke, or pulmonary embolism, respectively. These people are given warfarin, an anticoagulant. Warfarin inhibits coagulation by preventing the recycling of vitamin K and creating vitamin K deficiency (Fig. 5.2, Wallin and Martin, 1985). Inadequate γ -carboxylation of vitamin K-dependent coagulation proteins interferes with coagulation cascade, and inhibits blood clot formation. Experts advise a reasonably constant dietary intake of vitamin K that meets current dietary recommendations (60–80 mcg/day) for patients on vitamin K antagonists, like warfarin (Booth, 1999).

Three vitamin K-dependent proteins, namely osteocalcin, MGP, and protein S have been isolated from bones. Osteocalcin is synthesized by osteoblasts that presumably play a role in bone mineralization. The mineral binding capacity of osteocalcin requires γ -carboxylation of three glutamic acid residues. MGP found in bone, cartilage, and soft tissue, including blood vessels is known to prevent the calcification of soft tissue and cartilage, while facilitating normal bone growth and development. The observation that a knockout of MGP leads to complete calcification of the arteries by two months of age (Luo *et al.*, 1997) indicates the importance of Gla-proteins other than blood coagulation factors. The protein S is synthesized by osteoblasts for unknown function. Children with inherited protein S deficiency are known to suffer from increased blood clotting and decreased bone density (Booth, 1997). Vitamin K deficiency and long-term warfarin treatment have been found to be associated with osteoporosis and risk of hip fractures (Caraballo *et al.*, 1999; Vergnaud *et al.*, 1997).

The GGCX enzyme catalyzes γ -carboxylation of proteins important for blood coagulation and bone metabolism as described above (Furie *et al.*, 1999). GGCX essentially requires vitamin K hydroquinone to catalyze γ -carboxylation of glutamic acid residues. It is suggested that NQO1 catalyzes metabolic reduction of vitamin K to vitamin K hydroquinone that is made available to GGCX for carboxylation reactions. This suggestion is based on several observations and published reports including some from our laboratory. The various published reports have clearly shown that NQO1 catalyzes two-electron reduction of menadione (vitamin K3) to menadione hydroquinone (Jaiswal *et al.*, 1988; Robertson *et al.*, 1986). This along with structural analogy between vitamin K1, K2, and K3 indicates that vitamin K1 and K2 are also metabolized by NQO1 to vitamin K hydroquinone. However, studies suggest that NQO1 is only a minor player in the reduction of vitamin K1 and K2 to vitamin K hydroquinone (Preusch and Smalley, 2006). In addition, the NQO1-null mice generated in our laboratory do not have bleeding problems (Jaiswal, unpublished data).

These together suggest that other enzymes such as vitamin K epoxide reductase or yet unknown enzymes catalyze metabolic conversion of vitamin K1 and K2 to hydroquinone. Is it possible that longer side chains, composed of a variable number of unsaturated isoprenoid residues, reduce the affinity of vitamin K1 and K2 for NQO1? Further studies are required to explore the enzymes that catalyze metabolism of vitamin K1 and K2 to hydroquinone. NQO2, like NQO1, also catalyzes reduction of vitamin K3. The role of NQO2 in reduction of vitamin K1 and K2 is expected but remains unknown.

IV. QUINONE OXIDOREDUCTASES AND METABOLIC DETOXIFICATION OR ACTIVATION OF VITAMIN K

Vitamin K is a redox cycling compound. Most of the studies were focused on vitamin K3 or menadione. Menadione undergoes either one-electron reduction, producing semiquinone radicals, or two-electron reduction, resulting in hydroquinone (Fig. 5.3, Jaiswal, 1988; Robertson *et al.*, 1986). One-electron reduction of menadione is catalyzed by NADPH:cytochrome P450 reductase and other enzymes. The semiquinone in the presence of oxygen is oxidized back to menadione. This leads to reduction of oxygen or generation of ROS. Supporting this theory is the observation that menadione increased oxidative stress that led to increased DNA strand breaks (Nutter *et al.*, 1992; Ross *et al.*, 1986). This was further supported by observations that antioxidants such as glutathione and enzymes, like catalase and superoxide that quench ROS, decreased the oxidative stress of menadione (Sun *et al.*, 1997). On the other hand, NQO1 uses NADH or NADPH as electron donors and catalyzes two-electron reduction of menadione to menadione hydroquinone (Jaiswal, 1988; Robertson *et al.*, 1986). This pathway of reduction of menadione does not generate unstable semiquinone and ROS. In *in vivo* setup, NQO1 competes with P450 reductase to metabolize menadione and like quinones (Joseph and Jaiswal, 1994). In other words, two-electron reduction of menadione leads to detoxification of menadione and protection against oxidative stress-induced adverse effects. The support for this came from the studies on NQO1-deficient NQO1-null mice. NQO1-null mice demonstrated increased menadione toxicity, as compared to wild-type mice (Radjendirane *et al.*, 1998). This was evident from menadione concentration-dependent increase in liver-specific enzyme, alanine amino-transferase (ALT), in serum from NQO1-null mice, as compared to wild-type mice (Fig. 5.4, Radjendirane, 1998). NQO2 is a second member of the NQO family of proteins (Jaiswal *et al.*, 1990). It was expected that NQO2, like NQO1, should protect against

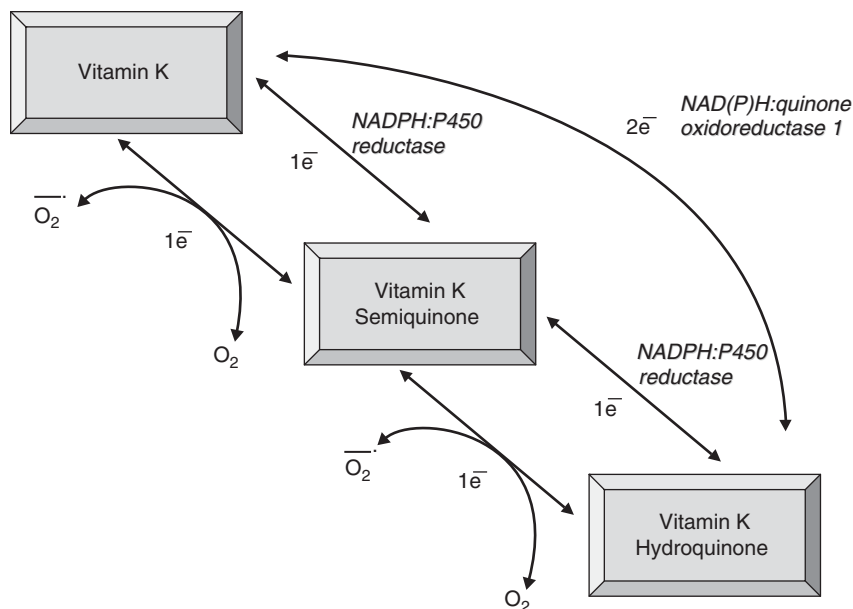


Figure 5.3 Redox cycling of vitamin K. Vitamin K is reduced by one-electron reducing enzyme cytochrome P450 reductase and NADPH to semiquinone that is either reduced by a second reaction to hydroquinone or is oxidized back to quinone by reducing oxygen. This cycle of conversion of vitamin K to semiquinone and back to quinone can result in generation of significant amount of reactive oxygen species (ROS) with adverse effects. NAD(P)H:quinone oxidoreductase 1 (NQO1) competes with P450 reductase and catalyzes two-electron reduction of vitamin K to hydroquinone, thus skipping the semiquinone and ROS generation.

menadione toxicity. Intriguingly, the studies with NQO2-null mice revealed just opposite results (Fig. 5.4, Long *et al.*, 2002b). Menadione treatment of NQO2-null mice resulted in protection against menadione-induced oxidative stress and hepatic toxicity. In other words, NQO2-null mice showed resistance to menadione-induced toxicity, as compared to wild-type mice. Increase in treatment with menadione did not lead to increased secretion of alanine amino-transferase in NQO2-null mice (Fig. 5.4, Long *et al.*, 2002b). Therefore, NQO1 and NQO2, two closely related enzymes differentially metabolize menadione. NQO1 metabolism of menadione results in detoxification of menadione and protection of cells against oxidative stress. On the contrary, NQO2 metabolism of menadione leads to activation and cytotoxicity. The NQO2 metabolic products of menadione remain unknown. Interestingly, there is no information available on the *in vivo* role of NQO1 and NQO2 metabolism of vitamin K1 and K2.

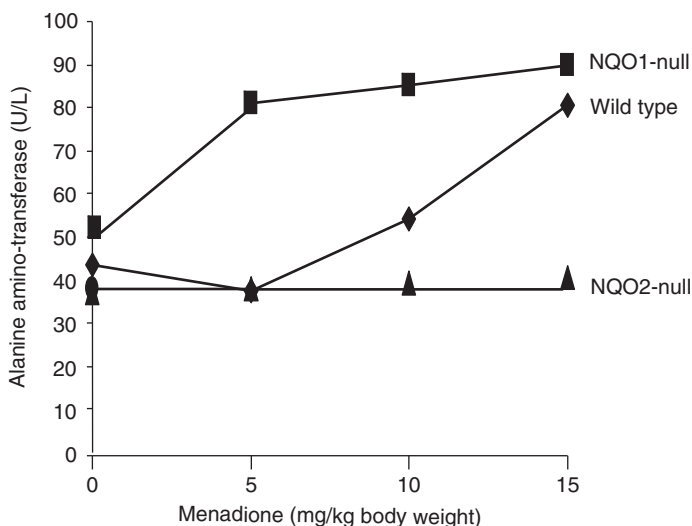


Figure 5.4 Menadione toxicity in wild-type, NQO1-null, and NQO2-null mice. Mice were injected intraperitoneally with 0 (DMSO control), 5, 10, and 15 mg of menadione/kg body weight. Menadione was dissolved in DMSO. The various doses were injected once a day for three consecutive days. Blood was withdrawn 24 h after the last dose and serum level of alanine aminotransferase was analyzed. The values are $\pm$ SD of four to ten mice.

V. QUINONE OXIDOREDUCTASES AND ANTICANCER EFFECTS OF VITAMIN K

The growth inhibitory and cytotoxic effects of vitamin K have been demonstrated both *in vivo* and *in vitro*. Menadione, as a radiosensitizer, increased survival of bronchial carcinoma patients (Lamson and Steven, 2003; Mitchell *et al.*, 1965). Pretreatment of mice with menadione potentiated the action of radiation compared to control (Taper *et al.*, 1996). Menadione treatment also increased survival of rats bearing hepatoma (Parekh *et al.*, 1992). The anticancer activity of menadione has also been demonstrated in a number of *in vitro* studies using both rodent and human cancer cell lines (Akman *et al.*, 1985, 1987; Chlebowski *et al.*, 1985a,b; Juan and Wu 1993; Noto *et al.*, 1989; Nutter *et al.*, 1991; Prasad *et al.*, 1981; Su *et al.*, 1987; Waxman and Bruckner, 1982). Menadione was found to be synergistic with 5-fluorouracil, bleomycin, cisplatin, and decarbazine in human oral epidermoid carcinoma cell culture (Waxman and Bruckner, 1982). Menadione also demonstrated an additive effect when combined with other chemotherapeutic agents including mercaptopurine, cytarabine, hydroxyurea, VP-16, vincristine, doxorubicin, mitoxanthine, mitomycin C,

actinomycin D, and thiopeta (Nutter *et al.*, 1991). Vitamin K1 and K2 have also been shown to act as anticancer agents (Lamson and Steven, 2003). However, one major difference is that vitamin K1 and K2 are effective only in millimolar concentration as compared to micromolar concentration of vitamin K3. The mechanism of anticancer effects of vitamin K remains unknown. However, it is thought that redox cycling of vitamin K produces ROS that leads to cytotoxicity and tumor cell death. NQO2 and other enzymes that metabolically activate vitamin K are expected to contribute to anticancer action of vitamin K.

VI. FUTURE PERSPECTIVES

Future studies are required to clearly understand the metabolic reduction of vitamin K to vitamin K hydroquinone that plays a significant role in γ -carboxylation of specific glutamic acid residues leading to activation of blood clotting factors and factors in bone metabolism. The role of NQO1 in metabolic reduction of menadione (vitamin K3) to menadione hydroquinone is well established. However, studies are required to investigate the role of NQO1 and NQO2 in metabolic reduction of vitamin K1 and K2 to hydroquinone. Studies are also required to identify other enzymes such as VKOR in metabolic reduction of vitamin K to hydroquinone. The NQO1-null mice could be asset to investigate these aspects of vitamin K research. Future investigations should also focus on determination of the role of NQO1 and NQO2 in metabolic detoxification and activation of all three forms of vitamin K. The studies on NQO2 activation must be extended to elucidate the role of NQO2 in metabolic activation of vitamin K and its anticancer activity.

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STRUCTURE AND FUNCTION OF VITAMIN K EPOXIDE REDUCTASE

Jian-Ke Tie^{*} and Darrel W. Stafford^{*}

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Abstract

Vitamin K epoxide reductase (VKOR) is an integral membrane protein that catalyzes the reduction of vitamin K 2,3-epoxide and vitamin K to vitamin K hydroquinone, a cofactor required for the γ -glutamyl carboxylation reaction. VKOR is highly sensitive to inhibition by warfarin, the most commonly prescribed oral anticoagulant. Warfarin inhibition of VKOR decreases the concentration of reduced vitamin K, which reduces the rate of vitamin K-dependent carboxylation and leads to under-carboxylated, inactive vitamin K-dependent proteins. It is proposed that an active site disulfide needs to be reduced for the enzyme to be active. VKOR uses two sulfhydryl groups for the catalytic reaction and these two sulfhydryl groups are oxidized back to a disulfide bond during each catalytic cycle. The recent identification of the gene encoding VKOR allows us to study its structure and function relationship at the molecular level. The membrane topology model shows that VKOR spans the endoplasmic reticulum membrane three times with its amino-terminus residing in the lumen and the carboxyl-terminus residing in the cytoplasm. Both the active site (cysteines 132 and 135) and the proposed warfarin binding site (tyrosine 139) reside in the third transmembrane helix. VKOR is made at high levels in insect cells and is relatively easily purified. This should allow the determination of its three-dimensional structure. A detailed

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mechanism has been published and the purified enzyme should allow the testing of this mechanism. A major unanswered question is the physiological reductant of VKOR. © 2008 Elsevier Inc.

I. INTRODUCTION

K vitamins are a family of 2-methyl-1,4-naphthoquinone derivatives, that include the naturally occurring vitamin K₁, 2-methyl-3-phytyl-1,4-naphthoquinone; vitamin K₂, 2-methyl-3-polyisoprenoid-1,4-naphthoquinone; and the synthetic menadione (vitamin K₃) (Fig. 6.1).

Vitamin K₂ is comprised of a series of molecules with repeating unsaturated prenyl units in the 3 position of naphthoquinone of the molecule. The vitamin K₂ molecules with different side chains are called menaquinones-*n* (MK-*n*), where *n* stands for the number of the repeating prenyl units. MK4 is prepared synthetically, while MK7 is the most prevalent form in natto and MK9 is most prevalent in cheese. The relative amounts of vitamin K in foods was reported by Elder *et al.* (2006). In mammals, the main function of vitamin K is to act as a cofactor for the enzyme γ -glutamyl carboxylase (GGCX). GGCX catalyzes the posttranslational carboxylation of specific glutamic acid to γ -carboxyglutamic acid in a variety of vitamin K-dependent proteins (Presnell and Stafford, 2002). This posttranslational modification is critical for the biological functions of the vitamin K-dependent proteins that are involved in blood coagulation, bone and soft tissue mineralization, signal transduction, and cell proliferation (Berkner, 2005; Furie *et al.*, 1999; Vermeer, 1990). During the process of carboxylation, the γ -proton of the glutamic acid is abstracted by an oxygenated intermediate of vitamin K

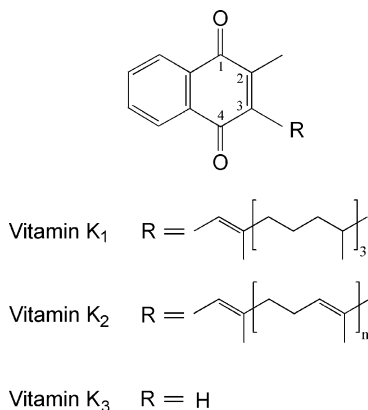


Figure 6.1 Chemical structure of K vitamins.

(Dowd *et al.*, 1995), followed by the addition of carbon dioxide. Concomitant with γ -glutamyl carboxylation, the reduced form of vitamin K [vitamin K hydroquinone (KH_2)] is oxidized to vitamin K 2,3-epoxide (KO), which because of the limited vitamin K amounts *in vivo*, must be converted back to KH_2 for the reaction to continue. This cyclic conversion of vitamin K establishes a redox cycle known as the vitamin K cycle (Oldenburg *et al.*, 2006; Stafford, 2005) (Fig. 6.2). Although hydroxy vitamin K was identified as a metabolite of KO (Fasco *et al.*, 1983a; Preusch and Suttie, 1984a), it is currently accepted that three forms of vitamin K are involved in the vitamin K redox cycle, that is, vitamin K, KH_2 , and KO. The enzyme responsible for the conversion of KO to its reduced form is vitamin K epoxide reductase (VKOR).

The epoxide form of vitamin K was discovered in 1970 as a metabolite of phylloquinone in warfarin-treated rats (Matschiner *et al.*, 1970). An enzymatic activity capable of converting KO back to vitamin K was reported by the same group later that year (Bell and Matschiner, 1970). It was proposed that warfarin administration caused the accumulation of KO in tissues and thus, increased the ratio of KO to vitamin K (Bell and Matschiner, 1972; Bell *et al.*, 1972). Because of its structural similarity to vitamin K, they assumed that the accumulated KO was a competitive inhibitor of vitamin K-dependent carboxylation. This hypothesis was soon questioned, however, because administration of high doses of KO to

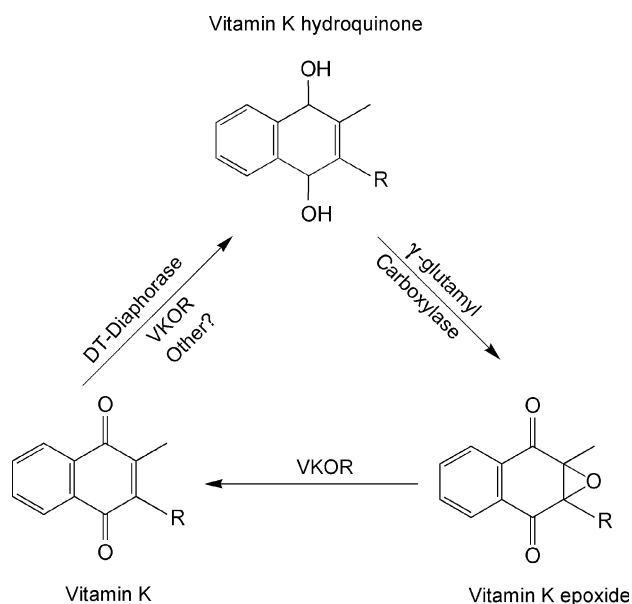


Figure 6.2 The vitamin K cycle.

normal or warfarin-resistant rats did not inhibit prothrombin synthesis (Goodman *et al.*, 1974; Sadowski and Suttie, 1974). Later, Fasco and Principe (1980) identified KH_2 , the substrate for γ -glutamyl carboxylase (Friedman and Shia, 1976; Sadowski *et al.*, 1976), as a metabolite of KO. Because warfarin inhibits the formation of KH_2 from both KO and vitamin K, they proposed that warfarin inhibition of VKOR was relevant to its anticoagulant activity. This hypothesis is now well accepted (Hallgren *et al.*, 2006; Sun *et al.*, 2005; Wajih *et al.*, 2005a).

In vitro, the enzymatic activity of VKOR requires a dithiol compound, such as dithiothreitol (DTT). Silverman (1981a) proposed that DTT, the commonly used reducing reagent in the *in vitro* activity assay, reduces a critical disulfide within the active site of VKOR that is reoxidized to a disulfide during the conversion of KO to vitamin K. VKOR is the target of the widely used anticoagulant warfarin which is proposed to exert its action by binding to the oxidized form of VKOR preventing the reduction of the essential cysteines to its active form (Fasco *et al.*, 1983b). In the past 35 years, all the functional studies on VKOR were based on microsomal preparations. To better understand the mechanism of VKOR function and the molecular basis of warfarin inhibition, it is necessary to have the purified enzyme. Recently, the gene encoding VKOR catalytic activity was identified independently by our laboratory (Li *et al.*, 2004) and that of Johannes Oldenburg (Rost *et al.*, 2004). We have purified the recombinant VKOR from insect cells (Chu *et al.*, 2006). These studies make it possible to begin to understand the structure and function relationship of VKOR at the molecular level.

II. PURIFICATION OF VKOR

Membrane proteins are naturally embedded and function in lipid bilayers and are generally not soluble in aqueous solution. Membrane lipids that form the bilayer can be important modulators of membrane protein structure and function (Lee, 2004; Opekarova and Tanner, 2003; Zhang *et al.*, 2005). Purification of the membrane protein requires the removal of the protein from the native membrane and its dispersal in solution. Purification of a membrane protein is usually more difficult than purifying a soluble protein since most membrane proteins lose their biological function in the absence of a proper lipid environment. The first step of membrane protein purification requires the solubilization of the membrane with a detergent that does not denature the protein but disperses the membrane (le Maire *et al.*, 2000; Silviu, 1992). Selection of the right detergent for each membrane protein is a critical step, as no single detergent is suitable for all membrane proteins and many detergents cause their irreversible denaturation.

Generally speaking, nonionic detergents are mild nondenaturing detergents which only break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions. This allows many membrane proteins to be solubilized in nonionic detergents without affecting their structure so that they can be isolated in their biologically active form (Seddon *et al.*, 2004; Silvius, 1992).

Solubilization of membrane proteins can be described by a three-stage model as detergent concentration increase (le Maire *et al.*, 2000). In the first stage, at low detergent concentration, detergent is taken up in nonmicellar form by the membrane phase without disrupting the membrane. As the detergent concentration increases, detergent-saturated membranes coexist with mixed lipid–detergent micelles. This is considered to be the second stage. In the third stage, membrane components are fully solubilized by uptake into detergent micelles. The hydrophobic membrane embedded region of the membrane proteins becomes enwrapped in a layer of protective detergent coating and the membrane proteins are considered to be in a solubilized state. Thus, the detergent–lipid–protein ratio is another important factor for the successful solubilization of membrane proteins.

Since the discovery of the enzymatic activity of VKOR in 1970 (Bell and Matschiner, 1970), numerous unsuccessful attempts to purify this integral membrane enzyme from hepatic microsomes were reported (Begent *et al.*, 2001; Cain *et al.*, 1997; Guenther *et al.*, 1998; Lee *et al.*, 1985; Wallin and Guenther, 1997). Purification of VKOR has proved exceptionally difficult because it is rendered inactive by any detergent that achieves its solubilization (Chu *et al.*, 2006; Hildebrandt *et al.*, 1984). Primarily because of the loss of activity that accompanied purification, it was postulated that reduction of KO was performed by a multienzyme complex (Begent *et al.*, 2001; Cain *et al.*, 1997; Guenther *et al.*, 1998; Lee *et al.*, 1985; Wallin and Guenther, 1997). Although nonionic detergents are considered to be mild detergents and are commonly used in membrane protein solubilization, the best detergent for hepatic VKOR microsome solubilization seems to be the relatively mild ionic bile acid salts (cholate or deoxycholate) (Lee *et al.*, 1985; Siegfried, 1978). It has been reported that 0.5% potassium cholate releases nearly 60% of the VKOR activity originally present in intact microsomes (Siegfried, 1978), while VKOR activity was dramatically decreased in the nonionic detergent Triton X-100 (Siegfried, 1978; Whitton *et al.*, 1978). The detergent, Deriphat 160, was found to be an efficient detergent for the solubilization of VKOR, but the enzyme was inactive in its presence. Removal of the detergent by Sephadex G25 gel filtration partially restored VKOR activity (Hildebrandt *et al.*, 1984). In addition, the zwitterionic detergent (3-[(3-cholamidopropyl)di-methylammonio]-1-propane sulfonate) (CHAPS) was also found to be a good detergent for VKOR microsome solubilization (Cain *et al.*, 1997; Mukharji and Silverman, 1985).

The first partial purification of VKOR from rat liver microsomes was reported by Lee *et al.* (1985). Sodium cholate solubilized rat hepatic microsomes were separated by a discontinuous sucrose gradient to isolate a 200S microsomal sub-fraction that contained VKOR activity. This partially purified VKOR has 2.5–3.0 times higher specific activity for the KO to vitamin K and vitamin K to KH_2 conversions than the starting microsome. Attempts to further purify VKOR from the 200S fraction resulted in loss of enzymatic activity. They observed a 14–17 kDa warfarin-sensitive protein (WSP) in the 200S fraction which was labeled by chemical modification of the free cysteine residues using radioactive N -[^3H]ethylmaleimide ([^3H]NEM). The substrates, KO and vitamin K, and the inhibitor, warfarin, all effectively blocked the incorporation of [^3H]NEM to reduced WSP. Based on their results, Lee *et al.* (1985) suggested that the [^3H]NEM-labeled component is a single polypeptide containing one disulfide bond that exhibits VKOR activity. Unfortunately, these authors finally concluded that it is unlikely that a single species of this relative low molecular weight could catalyze these complex reactions. The recent purification of active recombinant VKOR as an 18-kDa enzyme (Chu *et al.*, 2006) agrees well with the observations from Fasco's laboratory (Lee *et al.*, 1985). The purified enzyme employs a CXXC redox center as the active site for KO reduction (Wajih *et al.*, 2005b).

Since it was generally accepted that VKOR is a multienzyme complex, purification of VKOR focused on searching for the potential component(s). In searching for the potential protein components that could be involved in the KO reduction system, Cain *et al.* (1997) subjected CHAPS-solubilized rat liver microsomes to a Bio-Gel P-100 gel-filtration column. Enzymatic activity was lost but some activity could be regained by combining the flow-through and retained fractions. The flow-through fraction was further subjected to a cellulose DE52 anion exchange column. The unbound fraction from the DE52 column also restored VKOR activity. These authors did amino-terminal sequencing of this mixture of proteins and identified several members of the glutathione-S-transferase (GST) gene family. These GSTs were further purified by an agarose–glutathione affinity column and they were proposed to be the warfarin-sensitive component of the VKOR enzyme complex in the ER membrane (Cain *et al.*, 1997). Further results from this group suggested that microsomal epoxide hydrolase, an integral membrane protein, is the second component of VKOR complex (Guenther *et al.*, 1998; Wallin and Guenther, 1997). Based on their results, these authors proposed that dimeric GST provided the warfarin-sensitive thiol redox center while the microsomal epoxide hydrolase provided the KO binding site for the catalytic reduction reaction (Guenther *et al.*, 1998; Wallin and Guenther, 1997).

Further support for the hypothesis that VKOR is a complex of at least two components was provided by Begent *et al.* (2001). These authors used

a series of ion-exchange chromatography and size exclusion gel filtration columns to purify a warfarin-insensitive component which possesses the activity of reducing KO to vitamin K. An unknown factor, which was referred to as “warfarin binding factor,” was found to be present in the void volume fraction of the gel filtration. Although these results support the hypothesis that the VKOR complex possesses one warfarin-insensitive catalytic site for the conversion of KO to vitamin K, and another warfarin-sensitive thiol redox center which binds the exogenous (DTT) and endogenous reductant, it is difficult to rationalize that “warfarin-sensitive factors” found by Begent *et al.* is GST, as identified by Cain *et al.* (1997), since the molecular weight of these two are much different. In addition, we do not know whether the warfarin-sensitive fraction which possesses VKOR activity found by these authors is the microsomal epoxide hydrolase identified by Cain *et al.* (1997) since the identity of the protein is unavailable. However, it was later shown that a mouse knockout of microsomal epoxide hydrolase had no defect in vitamin K metabolism (Miyata *et al.*, 1999), indicating that microsomal epoxide hydrolase is not involved in KO reduction.

Interestingly, in the attempts to purify VKOR from bovine liver microsomes, Mukharji and Silverman (1985) reported the purification of homogeneous warfarin-insensitive VKOR that reduces KO to hydroxy vitamin K. However, they concluded that this enzyme is different from the warfarin-sensitive VKOR that reduces KO to vitamin K. Bovine liver microsomes were solubilized by 1% CHAPS and the warfarin-insensitive VKOR was purified by four chromatographic steps: PBE-94 ion exchanger, hydroxylapatite, DEAE-cellulose, and Sephacryl S-200 gel filtration. The purified enzyme was assumed to be a dimer with identical subunits and a molecular weight of 25 kDa. This warfarin-insensitive enzyme only used DTT as a reducing agent, but not 2-mercaptoethanol, reduced glutathione, 1,2-ethanedithiol, or reduced lipoic acid like warfarin-sensitive VKOR. While this enzyme is apparently not VKOR, it was an important work and it would be interesting to repeat these studies to determine if the enzyme they purified is related in any way to VKOR.

One common conclusion drawn from previous purification attempts is that VKOR is a multienzyme complex. However, after the identification of the VKOR gene (Li *et al.*, 2004; Rost *et al.*, 2004), it was found to code for a 163-amino acid integral membrane protein that contained a CXXC motif characteristic of the thioredoxin family of enzymes. This enzyme was designated VKOR by us and VKORC1 (complex subunit 1) by Oldenburg and his coworkers (Rost *et al.*, 2004), who assume that there is another subunit still to be identified. However, we were able to purify VKOR to >93% purity (Fig. 6.3) from insect cells (Chu *et al.*, 2006); moreover, all of the contaminating proteins were identified by mass spectrometry and are unlikely to contribute to VKOR activity. Furthermore, this single peptide enzyme can accomplish both the conversion of KO to vitamin K and the

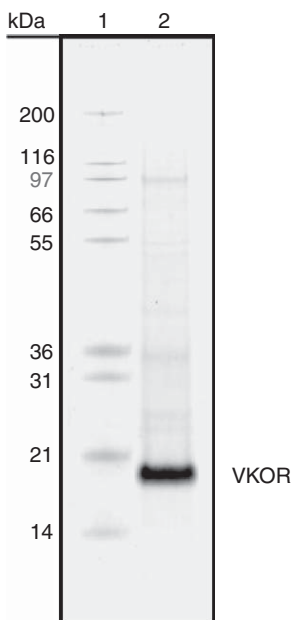
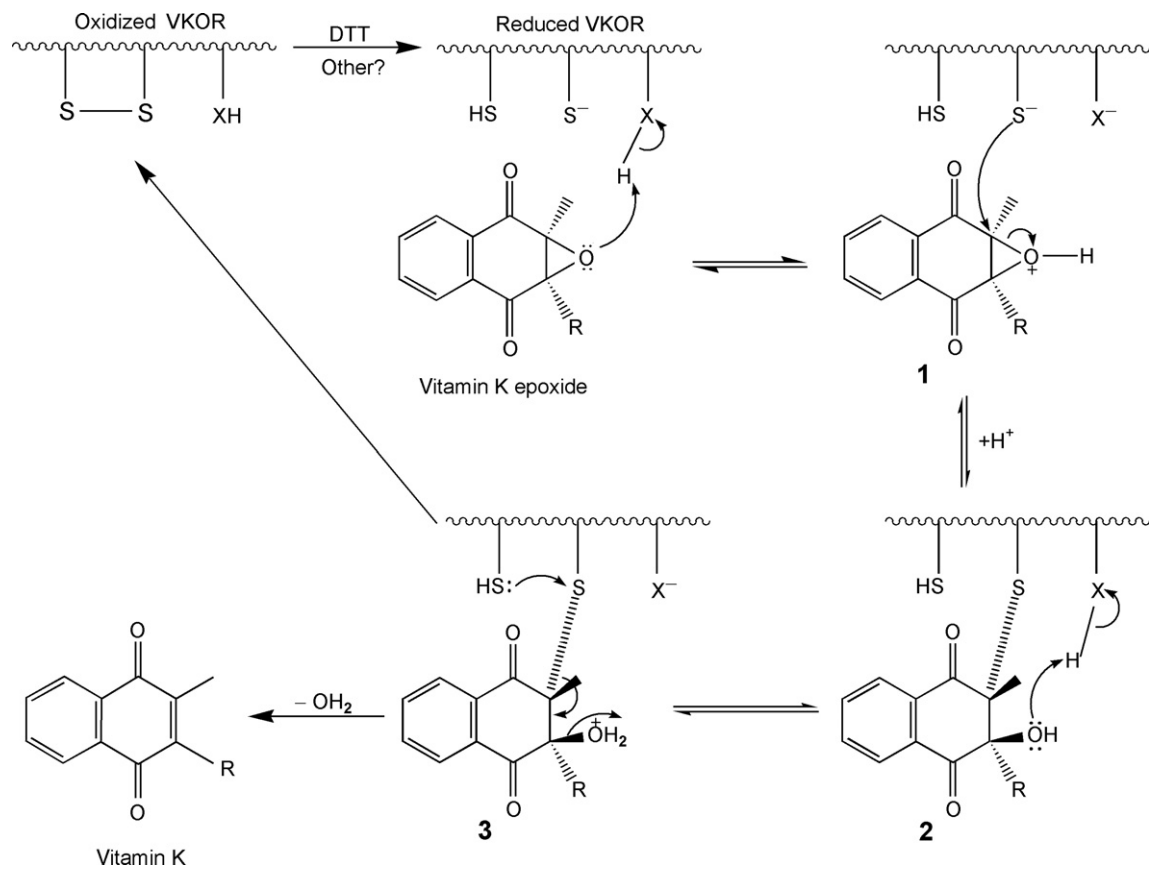


Figure 6.3 Affinity-purified recombinant VKOR expressed in insect cells. Lane 1, protein standard marker; lane 2, affinity-purified VKOR.

conversion of vitamin K to KH_2 (Chu *et al.*, 2006). Our result suggests that VKOR is a single enzyme, but it does not exclude the possibility that an unknown physiological reductant could associate with it to form an enzyme complex *in vivo*.

III. MECHANISM OF VITAMIN K EPOXIDE REDUCTION CATALYZED BY VKOR

In vitro reduction of KO by VKOR requires DTT to reduce the disulfide bond formed during each reaction cycle (Zimmermann and Matschiner, 1974). Based on a chemical model study, a reaction mechanism for converting KO to vitamin K by VKOR was proposed by Silverman (1981a) (Scheme I). In this model, an active site disulfide bond of VKOR is reduced by a thiol to activate the enzyme. KO binds to the reduced form of VKOR and the epoxide oxygen is protonated (compound 1) by a nearby acidic residue. One of the sulfhydryls in the active site attacks the carbon at position 2 of KO forming a β -hydroxy sulfide adduct (compound 2). Protonation of the β -hydroxyl group then generates an active leaving group for reductive elimination (compound 3) which is promoted by



Scheme I Proposed mechanism for vitamin K epoxide reduction catalyzed by VKOR.

another active-site sulfhydryl group. In this reaction, KO is reduced to vitamin K and the active site cysteines are oxidized to a disulfide.

A quantum chemical study of the reaction mechanism of KO reduction by VKOR supports and extends the Silverman mechanism (Davis *et al.*, 2007). It shows that once a key disulfide bond of VKOR is broken, the reaction is energetically favorable. The only difference this study suggests is that the source of the proton that protonates the epoxide oxygen is likely from an active site sulfhydryl group rather than from a nearby acidic residue as proposed by Silverman (1981a).

Data supporting the chemical model described above comes from the study of VKOR activity in rat hepatic microsomes with a variety of thiol compounds and sulfhydryl reactive reagents (Lee and Fasco, 1984). VKOR enzymatic activity was more efficiently stimulated by dithiol than by monothiol compounds. The only apparent structural requirement for an efficient reductant is the presence of two thiol groups in one compound. The distance between the two thiol groups appears unimportant (Lee and Fasco, 1984). The authors of this study suggest that monothiols are inefficient because a major proportion of the mixed disulfide, initially formed by reaction between the monothiol and the VKOR disulfide, undergoes reoxidation before a second molecule of monothiol can react. Dithiol compounds are believed to be more efficient because of the proximity of the second sulfhydryl group which cleaves the mixed disulfide at a rate that is sufficiently rapid to produce more fully reduced enzyme (Lee and Fasco, 1984). Additional evidence that an active site disulfide bond must be reduced before VKOR enzymatic activity appears is provided by inactivation of VKOR by the sulfhydryl reactive reagent *N*-ethylmaleimide (NEM). NEM inhibits VKOR activity much more efficiently if microsomes are pretreated with DTT. Moreover, inactivation of DTT-pretreated microsomes is effectively protected by vitamin K substrate. These results were confirmed with partially purified VKOR (Lee *et al.*, 1985). The mechanism of substrate protection from NEM inhibition was proposed as a result of the conversion of active site sulfhydryl groups to disulfides during the KO to vitamin K reactions. Wallin *et al.* (1987) reported that pretreatment of rat and human microsomes with DTT resulted in a greater inhibition by NEM of human microsomes than rat microsomes. They speculated that a larger pool of human enzymes were in an oxidized form. By using reductants with different hydrophobicity, Wallin *et al.* (2002) reported that the active site disulfide bond is located in a hydrophobic environment. All of these results are consistent with a mechanism where reduction of a disulfide bond in VKOR is essential for its enzymatic activity, and reduction of the KO oxidizes the active site sulfhydryl back to disulfide.

The requirement for breaking an active site disulfide bond of VKOR to initiate its enzymatic activity stimulated the search for its physiological reductant. Reduced glutathione was found to be only 6% as active as DTT for the reduction of KO by VKOR (Lee and Fasco, 1984). Although reduced lipoic

acid can significantly stimulate rat liver microsomal VKOR activity (Lee and Fasco, 1984; Thijssen *et al.*, 1994; Whitlon *et al.*, 1978), lipoic acid normally functions in its oxidized form as an oxidizing and acyl group transfer cofactor rather than as a reducing cofactor (Silverman and Nandi, 1988). Thijssen *et al.* (1994) reported that microsomes contain reduced nicotinamide adenine dinucleotide (NADH)-dependent lipoamide reductase activity. The reduced lipoamide stimulates microsomal VKOR activity with kinetics comparable with those of the DTT, and is warfarin sensitive. Since warfarin has no direct effect on lipoamide reductase activity, they proposed that microsomal lipoamide reductase is associated with VKOR in the membrane and microsomal lipoamide reductase is capable of transferring the reducing equivalents from NADH via reduced lipoamide to VKOR (Thijssen *et al.*, 1994).

Thioredoxin can serve as a physiological reductant of VKOR. If KO is used as a substrate for GGCX, thioredoxin stimulates γ -glutamyl carboxylation; however, in these experiments, thioredoxin was only 40% as efficient as DTT (Johan *et al.*, 1987). Silverman and Nandi (1988) confirmed that the reduced form of thioredoxin is the active form for the VKOR reaction since omission of thioredoxin reductase or reduced nicotinamide adenine dinucleotide phosphate (NADPH) (both required to reduce thioredoxin) completely destroy thioredoxin-dependent VKOR activity. In addition, Gardill and Suttie (1990) reported that thioredoxin serves as a reductant for VKOR in both the KO to vitamin K and vitamin K to KH_2 reactions. Further evidence supporting thioredoxin as a physiological reductant for VKOR comes from the work of Soute *et al.* (1992). These authors reported that protein disulfide isomerase (PDI) enhances the thioredoxin-driven VKOR reaction about tenfold, and that reduced RNAase could replace NADPH as a hydrogen donor for the reaction. In spite of results supporting thioredoxin as the physiological reductant of VKOR, this hypothesis has been questioned by Preusch (1992) for the following reasons: (1) under low detergent concentrations (0.01% Emulgen 911), thioredoxin-dependent VKOR activity is almost negligible under the tested condition; (2) under conditions where thioredoxin-dependent VKOR activity is significant, the reaction is no more sensitive to salicylate inhibition than was the DTT-dependent reaction; (3) thioredoxin is predominantly a soluble cytoplasmic protein that would not have access to the luminal surface of the ER where VKOR is located in the intact cell. Recently, Wajih *et al.* (2007) repeated and extended the work of Soute *et al.* (1992) suggesting that PDI is the physiological reductant for VKOR. They reported that reduced RNAase-triggered γ -glutamyl carboxylation is inhibited by the PDI inhibitor bacitracin and also by the small interfering RNA (siRNA) silencing of PDI in HEK 293 cells. The problem with these conclusions is that even though a reaction can be catalyzed by these components *in vitro*, there is no proof that it is the physiological mechanism in the cell. Furthermore, these results need to be demonstrated with purified

components; thus, in our view, the natural physiological reductant for VKOR is still unknown.

It was originally proposed that reduction of KO to vitamin K was carried out by sulfhydryl-dependent VKOR (Hildebrandt *et al.*, 1984; Sherman and Sander, 1981) and that the conversion of vitamin K to KH_2 might be accomplished by a second enzyme. Certainly, there are enzymes that can convert vitamin K to KH_2 and can function as antidotal enzymes when vitamin K is given to patients with warfarin poisoning. The reduction of vitamin K to KH_2 can be accomplished by at least two reductases that differ in their cofactor requirements: NAD(P)H-dependent quinone oxidoreductase (NQO1, previously referred as DT-diaphorase) (Fasco and Principe, 1982b; Wallin and Suttie, 1981; Wallin *et al.*, 1978) and sulfhydryl-dependent reductase (VKOR) (Fasco and Principe, 1980; Fasco *et al.*, 1982; Preusch and Suttie, 1984b; Sherman and Sander, 1981; Whitton *et al.*, 1978). The sulfhydryl-dependent reduction of KO to vitamin K and vitamin K to KH_2 share the following characteristics: (1) both reactions require an exogenous thiol compound as cofactor; (2) both reactions use reduced thioredoxin as a reductant; (3) both reactions are sensitive to warfarin inhibition; (4) both reactions can be inactivated by sulfhydryl reactive reagent such as NEM; and (5) both substrates, vitamin K and KO, prevent NEM inhibition of DTT pretreated microsomal VKOR. It has been suggested that both reactions are catalyzed by one microsomal enzyme or a multienzyme complex (Gardill and Suttie, 1990; Lee and Fasco, 1984; Preusch and Smalley, 1990; Preusch and Suttie, 1984b). Our recent results with purified recombinant VKOR demonstrate that reduction of KO and vitamin K can be carried out by one enzyme (Chu *et al.*, 2006).

The next question is whether KO and vitamin K are reduced by the same active site in VKOR. Based on the observation that neither KO nor vitamin K diminish the reduction rate of either VKOR catalyzed reaction, Lee and Fasco (1984) suggest that reduction of KO and vitamin K are carried out at separate sites that function independently. Preusch and Suttie (1984b) and Wallin *et al.* (1987), on the other hand, reported that vitamin K inhibition of KO reduction by VKOR is observed only when the substrate and inhibitor are added in the correct order. Preusch and Suttie (1984b) suggested that the failure to observe inhibition of the KO to vitamin K reaction by vitamin K is because the substrates were found on different liposomes. They proposed that inhibition of the KO to vitamin K reduction by vitamin K is a competitive inhibition and that the competitive substrates are reduced at the same enzymatic site (Preusch and Smalley, 1990). By comparing the reduction of KO and vitamin K by hepatic microsomal VKOR of warfarin-susceptible and warfarin-resistance rats, Fasco *et al.* (1982) reported that the reaction sequence is KO to vitamin K to KH_2 . Vitamin K must attain a minimum concentration before it can serve as a substrate for

further reduction. These results suggest that both KO and vitamin K are reduced by the same active site in VKOR.

IV. IDENTIFICATION OF THE ACTIVE SITE OF VKOR

In the February 5th, 2004, issue of *Nature*, the gene encoding VKOR catalytic activity was identified independently by our laboratory (Li *et al.*, 2004) and that of Oldenburg (Rost *et al.*, 2004). We used siRNA to inhibit VKOR activity in a cell line expressing sufficient VKOR to be measurable. Warfarin-resistant proteins (Kohn and Pelz, 2000) and combined deficiencies of vitamin K-dependent proteins (Fregin *et al.*, 2002) were mapped at chromosome 16 at 50–65 cM. Within this region were 190 predicted coding sequences. We were convinced that VKOR was an integral membrane protein (Zimmermann and Matschiner, 1974). Therefore, we threaded the translated genes through transmembrane prediction programs and selected those unknown genes that were predicted to have transmembrane regions. We then made pools of siRNA of each of the 13 gene candidates, and examined the effects of each pool on the expression level of VKOR in cultured cells. Of the 13 siRNA pools, only one pool significantly reduced the intrinsic VKOR activity of the transfected cells. The putative VKOR gene was confirmed by cloning and overexpression in insect cells. The insect cells exhibited very low VKOR activity under the assay condition (Li *et al.*, 2004). In Oldenburg's study, they sequenced a large number of genes (in the 16p12-q21 region of human chromosome 16) from patients exhibiting combined deficiencies of vitamin K-dependent proteins. Two putative mutations were found in a gene which extended over 5,126 base pairs and was comprised of three exons encoding a protein of 163 amino acids with a calculated relative molecular mass of 18,000 Da. The gene was named *vitamin K epoxide reductase complex subunit 1* (VKORC1). Overexpression of the wild-type VKORC1 in HEK 293 cells resulted in a significant increase of warfarin-sensitive VKOR activity (Rost *et al.*, 2004). It was reassuring that both laboratories identified the same sequences as VKOR.

The cloning of the gene encoding VKOR allowed the beginning of the study of VKOR at the molecular level. As discussed above, sulfhydryl groups are expected to be the active site catalytic residues. There are seven cysteines in the VKOR sequence at positions 16, 43, 51, 85, 96, 132, and 135. Alignment of multiple sequences show that four cysteines at position 43, 51, 132, and 135 are absolutely conserved (Goodstadt and Ponting, 2004). These four cysteines, as well as a conserved serine or threonine at position 57, were proposed to be the active site residues of VKOR (Goodstadt and Ponting, 2004). Cysteines 132 and 135 comprise a

CXXC redox motif (characteristic of the thioredoxin family of enzymes) and are located within the third transmembrane helix near the luminal surface (Fig. 6.7) (Tie *et al.*, 2005). The hydrophobic location of cysteines 132 and 135 is consistent with the observation that the active site of VKOR is located in a hydrophobic environment (Wallin *et al.*, 2002). In addition, because the helix would presumably require a lipid membrane environment for its proper structure, the position of the active site relative to the membrane may explain the loss of VKOR enzymatic activity when the microsomal membrane is solubilized by detergent. Goodstadt and Ponting (2004) proposed that the CXXC motif in VKOR could perform the nucleophilic attack on KO carbonyl groups. To confirm this hypothesis experimentally, Wajih *et al.* (2005b) mutated both cysteines 132 and 135 to serine and expressed the VKOR mutants in BHK21 cells. Both of the VKOR mutants were inactive, further supporting the notion that the thioredoxin-like motif of CXXC in VKOR is the active site.

To further examine the role of all seven cysteines and the conserved residues at position 57 in VKOR, Rost *et al.* (2005) mutated each of these residues to serine or alanine individually and expressed the VKOR mutants in HEK 293 cells. VKOR activities of reducing KO to vitamin K in the whole-cell extracts were examined and compared with that of wild-type enzymes. Mutations of cysteines 132 and 135 abolished VKOR activity. This agrees with the results of Wajih *et al.* (2005b) and further supports the idea that the CXXC motif is the active site of VKOR. In addition, Rost *et al.* reported that mutations at cysteine 51 and serine 57 also resulted in complete loss of VKOR activity. By analogy to ferredoxin-NADP⁺ reductase and cytochrome P450C24, they proposed that serine 57 is the binding site of KO (Rost *et al.*, 2005).

More recently, we have shown that recombinant VKOR can be purified to near homogeneity and that this purified recombinant enzyme can reduce both vitamin K and KO (Chu *et al.*, 2006). These results prompted us to revisit the role of the conserved cysteines in VKOR by site-directed mutagenesis (Jin *et al.*, 2007). Our results, using purified enzymes, showed that cysteines 132 and 135 are essential for both the conversion of KO to vitamin K and the conversion of vitamin K to KH₂. Surprisingly, we found that conserved cysteines 43 and 51 appear to be unimportant for either reaction. VKOR, with cysteines 43 and 51 mutated to alanine, had slightly greater activity than the wild-type enzyme, while deletion of the entire loop from residue 43 to 51 resulted in a VKOR molecule with 85% activity. The rationale for deleting amino acids 43–51 is that while characterizing VKOR by mass spectrometry, we observed a disulfide bond formation between cysteines 43 and 51. Therefore, if this disulfide is real, one might be able to join residue 42 to residue 52 and maintain a structure similar to the molecule with the disulfide bond. The relatively high activity (85%) of the loop-deleted VKOR molecule supports the idea of disulfide linkage between

cysteines 43 and 51. However, since mutations of both cysteines 43 and 51 to alanine also resulted in a molecule with high activity, neither a disulfide bond between 43 and 51 nor a free sulfhydryl is required for activity. A caveat to the conclusion that cysteines 43 and 51 are not required for VKOR enzymatic activity is that we used DTT as a reductant for the active site disulfide between cysteines 132 and 135 in the *in vitro* activity assay. It is possible that cysteines 43 and 51 can reduce the disulfide between cysteines 132 and 135. This does not appear in the *in vitro* reaction with DTT.

According to the mechanism proposed by Silverman (1981a), an acidic residue near the active site is required to protonate the epoxide oxygen in order to initiate the catalytic reaction. By looking at the amino acid sequence and the membrane topology of VKOR, there is only one acidic acid residue, aspartic acid 130, near the CXXC redox center. To examine its role in the catalytic reaction, we mutated aspartic acid 130 to alanine. The mutant protein has essentially wild-type activity, suggesting that residue 130 is not essential for the enzyme's activity (Jin *et al.*, 2007). This result agrees with our quantum chemical study that suggests an active site sulfhydryl group protonates the epoxide oxygen and once the active site disulfide bond is broken, the reaction is energetically favorable (Davis *et al.*, 2007).

V. WARFARIN INHIBITION OF VKOR

Warfarin (3-(α -acetylbenzyl)-4-hydroxycoumarin) (Fig. 6.4) is a widely prescribed oral anticoagulation drug that targets VKOR. The mechanism of warfarin action is now believed to limit the availability of KH_2 regenerated from KO by VKOR. Inadequate amount of KH_2 , the essential cofactor for γ -glutamyl carboxylation, results in the production of an under-carboxylated form of coagulation factors that are biologically inactive. Thus, warfarin indirectly prevents the production of functional coagulation factors involved in the clotting cascade (Stafford, 2005). The binding of warfarin to VKOR is very tight, and it seems irreversible (Fasco and Principe, 1982a; Lorusso and Suttie, 1972). Due to its structural

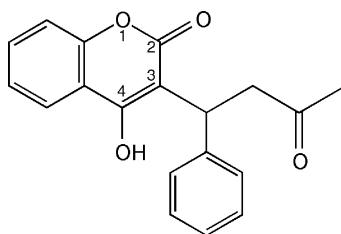
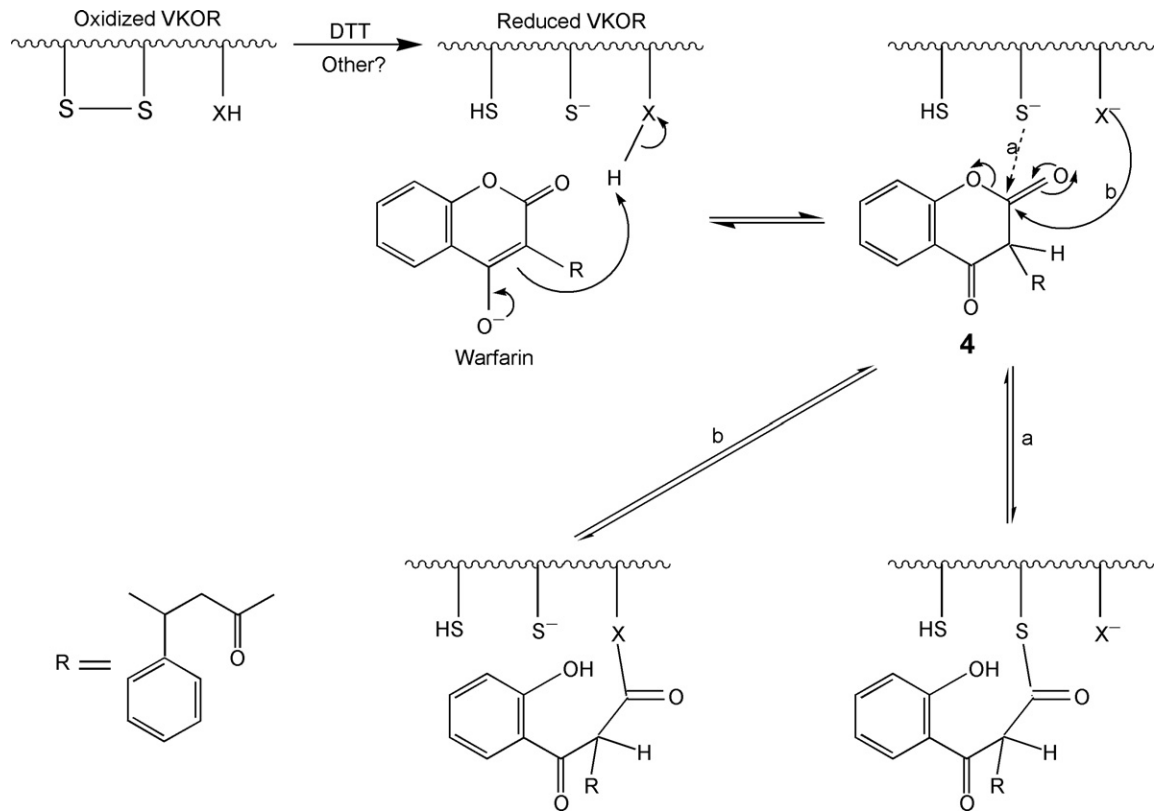


Figure 6.4 Chemical structure of warfarin.

similarity to vitamin K and based on the proposed mechanism of reduction of KO by VKOR, a chemical model for the mechanism of inactivation of VKOR by warfarin was also proposed by Silverman (1980, 1981b) (Scheme II). Similar to KO binding to reduced VKOR, Silverman proposed that deprotonated warfarin also binds to the reduced form of the enzyme. Protonation of the double bond in warfarin forms 2,4-chromandiones (compound 4), an unstable tautomeric form of warfarin. This compound is expected to be susceptible to a nucleophilic attack at the lactone carbonyl group that covalently modifies the VKOR active site by acylation of a sulfhydryl (pathway a) or the nearby nucleophile (pathway b).

Although the above proposed mechanism agrees with the irreversible characteristic of warfarin inhibition of VKOR, experimental data suggest that warfarin binds to the oxidized form rather than the reduced form of VKOR as proposed by Silverman (1980). It was reported that inactivation of VKOR by warfarin was greatest and occurred more rapidly when the enzyme was exposed to warfarin prior to its exposure to DTT (Fasco *et al.*, 1983b; Zimmermann and Matschiner, 1974). Moreover, preincubation of VKOR with DTT prior to warfarin incubation diminishes the extent of warfarin inhibition of VKOR activity. These results suggest that inhibition of VKOR activity by warfarin arises from its binding to the disulfide form of the enzyme, thus preventing the formation of the free sulfhydryls requisite for activity (Fasco *et al.*, 1983b; Lee *et al.*, 1985). Fasco *et al.* (1983b) reported further evidence supporting the hypothesis that warfarin binds the disulfide form of the enzyme. They reported that warfarin inhibition is greater when VKOR is preincubated with 100 μM rather than 10 μM KO. The substrate effect was more pronounced if the enzyme was preincubated with DTT. Their interpretation was that at a higher concentration of substrate, more disulfide was formed, while the preincubation with DTT provided more active enzyme for the substrate to react with. As proposed in Scheme I, reduction of KO to vitamin K results in the transformation of the reduced form of the enzyme (active site sulfhydryl) to its oxidized form (active site disulfide). This oxidized enzyme can either be reduced by DTT to its active form or it can bind to warfarin, effectively removing it from the pool of active enzyme.

More recently, Gebauer (2007) synthesized a series of warfarin derivatives and examined the effects of these compounds on VKOR activity. Results show that inhibition of VKOR by warfarin depends on the deprotonation of its 4-hydroxy group. Changing the 4-hydroxy group (pK_a 4.85) to a sulfhydryl group (pK_a 6.60) results in an eightfold decrease of its ability to inactivate VKOR. When the 4-hydroxy group was replaced by nonionic groups, it lost the ability to inhibit VKOR activity. Moreover, the nature of the substituent on carbon 3 of warfarin modulates its inhibition of VKOR. Ferulenol (Fig. 6.5), a natural product from the plant, *Ferula communis*, which has an isoprenyl side chain similar to vitamin K on carbon 3, is



Scheme II Proposed mechanism of inactivation of VKOR by warfarin.

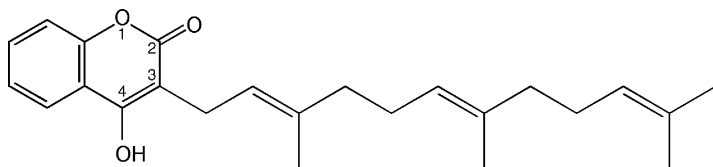


Figure 6.5 Chemical structure of ferulenol.

~22 times more efficient than warfarin as an inhibitor of VKOR. Based on these observations, it is proposed that deprotonated warfarin binds to the active site of the oxidized (disulfide) form of VKOR which mimics the transition state of the reductive elimination of substrate, 2-hydroxyvitamin K (compound **3** in [Scheme 1](#)) ([Gebauer, 2007](#)). These results support the notion that (1) warfarin binds to the oxidized form rather than the reduced form of VKOR; (2) warfarin and KO bind to the same rather than a different site on VKOR ([Fasco *et al.*, 1983b](#)); and (3) inhibition of VKOR by warfarin is not covalent.

The identification of the VKOR gene ([Li *et al.*, 2004](#); [Rost *et al.*, 2004](#)) makes it possible to understand the mechanism of warfarin inhibition at the molecular level. [Pelz *et al.* \(2005\)](#) identified three mutations, Y139C, Y139S, and Y139F, in warfarin-resistant rats and mice that correlate with warfarin resistance. Furthermore, recombinant VKOR with Y139C, Y139S, or Y139F mutations are warfarin resistant ([Pelz *et al.*, 2005](#); [Rost *et al.*, 2005](#)). Since the tyrosine residue in a Thr-Tyr-Ala motif has been identified as a dicoumarol binding site in NQO1 (which reduces quinone to hydroquinone) ([Ma *et al.*, 1992](#)), they proposed that the Thr-Tyr₁₃₉-Ala sequence in VKOR forms at least part of the warfarin binding site ([Oldenburg *et al.*, 2006](#)). Substitution of tyrosine 139 by phenylalanine results in a VKOR molecule that is resistant to warfarin but that has wild-type enzyme activity. Since the only difference between tyrosine and phenylalanine is the hydroxyl group of tyrosine, they proposed that this hydroxyl group plays an essential role in warfarin binding ([Rost *et al.*, 2005](#)). Since the publication of the article by [Rost *et al.* \(2005\)](#), a crystal structure of NQO1 in complex with its inhibitor dicoumarol has been published ([Asher *et al.*, 2006](#)). Indeed, the hydroxyl group of tyrosine in the dicoumarol binding site forms a hydrogen bond with the oxygen at position 5 of dicoumarol. This result supports the above hypothesis of the importance of VKOR tyrosine hydroxyl group in warfarin binding. In addition, an α -helix wheel representation of the third transmembrane domain (TMD) of VKOR ([Fig. 6.6](#)) shows that tyrosine 139 aligns on the same side of the helix as the two active site cysteines, and that it is one turn beyond the CXXC redox center. The position of the proposed Thr-Tyr₁₃₉-Ala warfarin binding motif relative to the CXXC active site motif supports the

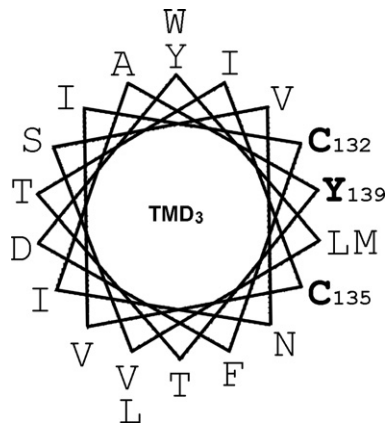


Figure 6.6 α -Helix wheel alignment of the third TMD of VKOR.

hypothesis that the inhibitor (warfarin) and substrate (KO) bind to the same site in VKOR.

Except for the “hot-spot” mutations at residue 139 (Pelz *et al.*, 2005), V29L, D36Y, V45A, R58G, V66M, and L128R, mutations in VKOR have been identified in warfarin-resistant patients (D’Ambrosio *et al.*, 2007; Harrington *et al.*, 2005; Rost *et al.*, 2004). Since these mutations are widely spread in the VKOR sequence, it seems unlikely that all of these residues are involved in warfarin binding. On the other hand, it seems likely that these residues may affect the folding and/or the overall structure of VKOR. Interestingly and importantly, recent studies show that the dose of warfarin required for a therapeutic level of anticoagulation is linked to single nucleotide polymorphisms (SNPs) in VKOR (D’Andrea *et al.*, 2005; Li *et al.*, 2006; Wadelius and Pirmohamed, 2007; Yin and Miyata, 2007; Yuan *et al.*, 2005). The molecular mechanism for the correlation between warfarin dosage and VKOR polymorphisms may be related to mRNA expression levels and stability. Since 12% of patients given warfarin develop severe bleeding, often resulting in death, determining the status of the polymorphisms in a patient is important before initiating warfarin treatment. In a very important paper, Wang *et al.* (2006) examined the association of 1,811 stroke patients, 740 patients with heart disease, and 253 patients with aortic dissection. They reached the conclusion that cytosine at nucleotide 2255 confers almost twice the likelihood of having vascular disease. It is likely that the entire population should be examined for this VKOR phenotype, and that those people with low VKOR activity should have their diet supplemented with vitamin K.

VI. MEMBRANE TOPOLOGY OF VKOR

To better understand the structure and function relationships that define the activity of the integral membrane protein VKOR, it is necessary to know its membrane topology when no three-dimensional structure is available. Currently, several computer programs to predict the topology of membrane proteins are available (Melen *et al.*, 2003; Moller *et al.*, 2001; Taylor *et al.*, 2003). The reliability of the topology prediction for a given protein is increased if the predictions from a number of different programs agree. If the location of one of the termini of a membrane protein is known, the best current program can correctly predict the topology of 65–70% of those membrane proteins with known crystal structure (Melen *et al.*, 2003). Moreover, using C-terminal reporter fusions, experimental examination of the membrane protein topology is very reliable (Kim *et al.*, 2003). Therefore, these computer programs are useful as a guide for experimental study of the membrane protein topology, that is, its specific number of transmembrane segments and their orientations relative to the cytoplasm or lumen of ER membranes (von Heijne, 2006).

We used topology prediction programs to predict the probable TMD regions in VKOR. Then, we determined the membrane topology of VKOR using *in vitro* translation/cotranslocation of intact VKOR and a series of VKOR truncations containing an N-linked glycosylation reporter tag (Tie *et al.*, 2005). Our results suggest that VKOR is a type III membrane protein with three TMDs (10–29, 101–123, and 127–149). The amino terminus resides in the lumen of the ER, whereas the carboxyl terminus resides in the cytoplasm (Fig. 6.7). The main features of this topology model were confirmed by experiments with microsomes derived from insect cells expressing full-length VKOR. One hydrophobic region (75–97), which is predicted to be a TMD, can serve as a stop-transfer sequence when used in isolation. However, in the intact enzyme, the membrane integration of this sequence appears to be suppressed by a following sequence. It seems clear that it is not an authentic TMD in full-length VKOR; due to its hydrophobicity, it maybe partially inserted into the membrane as found in other membrane proteins (Antignani and Youle, 2006). The N-terminal domain of VKOR, like many type III membrane proteins, is relatively short (nine amino acid residues), which facilitates its transfer into the lumen (Goder and Spiess, 2001).

The predominant topological determinant for the transmembrane orientation of integral membrane proteins is the asymmetric distribution of the charged residues on opposite sides of the ER membrane, the so-called “positive-inside rule” (von Heijne, 1989). The positive-inside rule means that positively charged residues are enriched on the cytoplasmic regions and are rarely found in the luminal loop of the membrane protein. Changing

even one charged residue that flanks the transmembrane helix can affect the overall orientation of a membrane protein (Rutz *et al.*, 1999). Once the membrane topology is determined, the folding of the multispanning integral membrane protein to its high-resolution three-dimensional structure mainly depends on the interactions between the transmembrane helices embedded in the membrane (Curran and Engelman, 2003; DeGrado *et al.*, 2003; Schneider *et al.*, 2007). This helix–helix interaction is frequently mediated by specific packing motifs. Recently, three motifs that are important for the helix–helix interactions have been characterized: the GxxxG motif, polar and hydrogen bonding motifs, and proline motifs (Senes *et al.*, 2004). There is an SxxxSxxSS polar and hydrogen bonding motif (Dawson *et al.*, 2002) in the second TMD of VKOR which could be important for helix–helix interactions and VKOR folding. Therefore, any factor that affects the membrane orientation of this TMD could result in the misfolding of VKOR or affect the interaction between VKOR and its potential counterparty protein components.

Rost *et al.* (2004) have identified a L128R mutation in warfarin-resistant patients and a R98W mutation in two vitamin K-dependent clotting factor deficiency type 2 families. Recombinant expression of these two VKOR mutants in HEK 293 cells only show slight VKOR activities above background (<9% activity compare with that of the wild-type enzyme) (Rost *et al.*, 2004). As shown in (Fig. 6.7) arginine 98 is one of the two positive charged residues that reside in the cytoplasm flanking the second TMD.

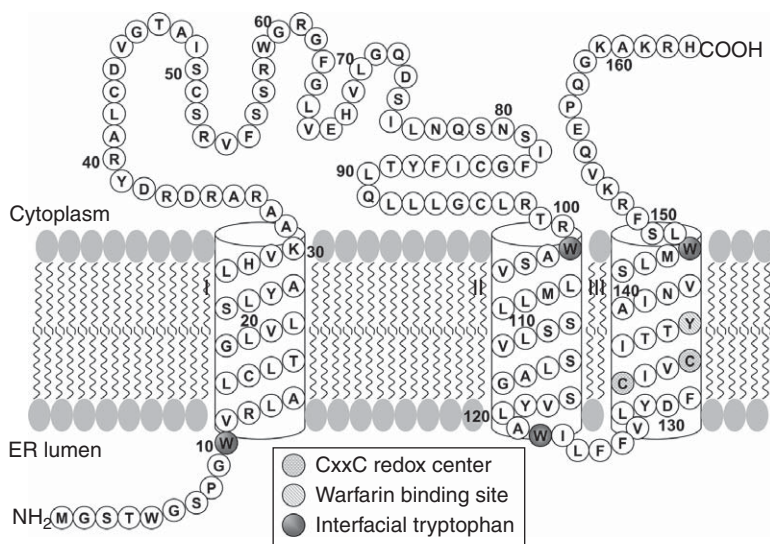


Figure 6.7 Proposed membrane topology of VKOR. Active site cysteines, warfarin binding tyrosine, and interfacial tryptophans are noted with different hatchmarks.

Therefore, changing of this positively charged residue to an aromatic residue could change the membrane orientation and integration of the second TMD, resulting in the disruption of the overall membrane topology and misfolding of VKOR. Similarly, L128R introduces a positively charged residue near the luminal surface of the third TMD, where the CXXC active site is located; this again would violate the “positive-inside rule.” Therefore, the significant decrease of VKOR activity in these two mutant proteins is likely due to the misfolding of VKOR.

In our VKOR topology model, the CXXC redox center of VKOR is located close to the luminal surface of the ER membrane in the third transmembrane helix. As we know, the activity of vitamin K-dependent carboxylase resides in the lumen of the ER; therefore, the luminal surface location of the CXXC active site motif of VKOR makes it possible to reduce the KO right after it is produced from the carboxylation reaction. The hydrophobic environment of the CXXC redox center (buried in the membrane) is also consistent with the hydrophobicity of its substrates KO and vitamin K. In addition, there is an aromatic residue rich sequence (*FFVLYDF*) right before the CXXC motif. These aromatic residues may form a multiplane binding pocket for the flat plane of the aromatic ring of vitamin K.

Another feature in this topology model of VKOR is the distribution of tryptophan residues in the interfacial regions of the membrane bilayers, as observed in other membrane proteins (Wallace and Janes, 1999). It is reported that tryptophan residues exhibit a strong tendency to remain within the interfacial region serving as anchor residues that fix the transmembrane helix within the lipid bilayer (Braun and von Heijne, 1999; de Planque *et al.*, 2003; Ridder *et al.*, 2000; Yau *et al.*, 1998). Moreover, it has been suggested that tryptophan residues support transmembrane helix–helix interactions (Ridder *et al.*, 2005). There are six tryptophans in VKOR, and four of them are located in the membrane bilayer interface. Tryptophans 101 and 122 are located in the cytoplasmic and luminal surfaces of the second TMD, respectively. They may serve as anchors to fix this TMD, which has the SxxxSxxSS polar and hydrogen bonding motif to interact with other TMDs in VKOR or with other proteins within the lipid membrane. Whether tryptophans serve as topology determinants, support transmembrane helix–helix interactions, or serve as critical residues for substrate binding in VKOR needs to be determined.

VII. CONCLUSION

VKOR is an integral membrane protein of the vitamin K cycle, and is the main target of the widely prescribed anticoagulant warfarin. It catalyzes the reduction of KO to its reduced form which serves as the cofactor for

vitamin K-dependent carboxylation of a variety of vitamin K-dependent proteins, including coagulation factors. Since the discovery of the enzymatic activity of VKOR, a large volume of work has been done to understand the mechanism of KO reduction and warfarin inhibition. Recently, the identification of the VKOR gene makes it possible to understand the structure and function of VKOR at the molecular level. Significant questions remain. What is the physiological reductant of VKOR? If two sulfhydryls are involved in the catalytic reaction (cysteines 132 and 135), which one attacks KO first? How do the natural mutations found in patients affect the structure and function of VKOR?

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VITAMIN K-DEPENDENT CARBOXYLATION

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Abstract

Vitamin K-dependent (VKD) protein carboxylation uses vitamin K epoxidation to convert Glus to carboxylated Glus (Glas), rendering VKD proteins active in physiologies that include hemostasis, apoptosis, bone mineralization, calcium homeostasis, growth control, and signal transduction. Clusters of Glus are modified by a processive carboxylase, generating a calcium-binding module that allows binding to either hydroxyapatite in the extracellular matrices or cell surfaces where anionic phospholipids become exposed, for example, during apoptosis or cell activation. Naturally occurring carboxylase mutations have been informative for function and are associated with bleeding complications and, surprisingly, a pseudoxanthoma elasticum (PXE)-like phenotype. A major advance in defining carboxylase function is the identification of the base that initiates carboxylation, which raises interesting possibilities for how vitamin K epoxidation is regulated by Glu substrate and carboxylase membrane topology. Vitamin K oxidoreductase (VKOR), the target of warfarin, generates the reduced vitamin K cofactor used by the carboxylase. Oxidation of active site thiols during vitamin K reduction inactivates VKOR, and activity is regenerated by an unknown reductase. The amounts of reduced vitamin K limit the capacity for carboxylation in cells, and overexpression of VKOR, but not carboxylase, improves

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carboxylation. However, the effect of VKOR overexpression is small, possibly because the reductase that regenerates VKOR activity is saturated. The review discusses these advances, as well as the potential impact of secretory components on carboxylation, which occurs during VKD protein secretion. Also discussed is the role of the carboxylase in mammals and lower organisms, including the bacterial pathogen *Leptospira interrogans* that has acquired a VKD carboxylase by horizontal transfer. © 2008 Elsevier Inc.

I. VITAMIN K-DEPENDENT PROTEIN FUNCTION

Vitamin K was discovered in association with a bleeding defect, and half of the 14 known human vitamin K-dependent (VKD) proteins are circulating hemostatic blood proteins (factors VII, IX, and X, prothrombin, and proteins C, S, and Z). Liver is the main site of hemostatic VKD protein synthesis, but several of these proteins are also expressed in other tissues and some have been shown to have additional roles besides hemostasis, for example, protein C and protein S in inflammation (reviewed in [Berkner and Runge, 2004](#)). Non-hemostatic VKD proteins include osteocalcin, an abundant bone matrix protein thought to be important to mineralization, and Gas6, a ligand for tyrosine kinase receptors (i.e., Axl, Sky, and Mer) that has multiple functions including growth control, chemotaxis, and apoptosis ([Fridell et al., 1998](#); [Goruppi et al., 1997](#); [Manfioletti et al., 1993](#); [Nakano et al., 1996](#)). Another nonhemostatic VKD protein is matrix Gla protein, which inhibits calcification. Mice bearing a deletion of the matrix Gla protein gene develop severe arterial calcification that ultimately leads to death ([Luo et al., 1997](#)). Humans lacking functional matrix Gla protein show calcification of cartilage but do not exhibit the gross vascular calcification observed in mice ([Munroe et al., 1999](#)). A family of four transmembrane Gla proteins identified by their homology to VKD proteins are suggested by structure to have a role in signal transduction ([Kulman et al., 1997, 2001](#)); however, their functions are not yet known. Finally, the carboxylase itself is also carboxylated ([Berkner and Pudota, 1998](#)), which may regulate the carboxylation of other VKD proteins. Many of the VKD proteins (e.g., the transmembrane Gla proteins, matrix Gla protein, and Gas6) show widespread tissue distribution, and virtually every tissue expresses VKD carboxylase activity. This distribution and the diverse range of functions for VKD proteins therefore implicates a broad biological impact of vitamin K. A single VKD carboxylase is responsible for carboxylating all VKD proteins, and deletion of the carboxylase gene results in embryonic lethality in mice ([Zhu et al., 2007](#)).

The requirement of carboxylation for function has been established more thoroughly for some VKD proteins than for others. Compelling evidence that the activity of hemostatic VKD proteins depends upon

carboxylation includes extensive biochemical characterization, clotting deficiency in patients with mutations that impair carboxylation (Brenner *et al.*, 1998; Darghouth *et al.*, 2006; Rost *et al.*, 2004b; Spronk *et al.*, 2000), and mutagenesis studies (e.g., Ratcliffe *et al.*, 1993; Zhang *et al.*, 1992). Hemostatic control is essential for life but clinical deficiency of hemostasis is rarely observed in the adult population, indicating that dietary vitamin K levels are sufficient in supporting this process. In contrast, undercarboxylation of osteocalcin is common (Binkley *et al.*, 2000), possibly because of differences in tissue utilization of vitamin K. However, the physiological consequence of undercarboxylation is unknown: carboxylation promotes osteocalcin binding to hydroxyapatite *in vitro* (Poser and Price, 1979), but no obvious skeletal abnormalities are observed when rats are administered warfarin (Price and Williamson, 1981), which inhibits vitamin K oxidoreductase (VKOR) and consequent carboxylation of VKD proteins. Determining whether carboxylation is required for osteocalcin activity is confounded by the fact that the role of this VKD protein has been difficult to assess, as exemplified by the mild phenotype of the osteocalcin null mice (Ducy *et al.*, 1996). In the case of matrix Gla protein, warfarin inhibition studies have been informative in indicating a requirement for carboxylation, as arterial calcification occurs in rats treated with warfarin (Price *et al.*, 1998). However, the extent of calcification is not as pronounced as in the null mice (Luo *et al.*, 1997; Price *et al.*, 1998), raising the question of whether uncarboxylated matrix Gla protein has some residual activity or whether the difference is due to incomplete inhibition by warfarin. Gas6 also appears to require carboxylation for function (Hasanbasic *et al.*, 2005; Nakano *et al.*, 1997; Stenhoff *et al.*, 2004). Early literature regarding this issue was confusing, in part because of the observation that a truncated molecule lacking the Gla domain binds tyrosine kinase receptor (Mark *et al.*, 1996). However, the naturally occurring form of Gas6 is a full-length molecule, and more recent studies with uncarboxylated versus carboxylated full-length Gas6 indicate that Glas are required for activity (Hasanbasic *et al.*, 2005; Nakano *et al.*, 1997; Stenhoff *et al.*, 2004). The Gla domain of Gas6 is thought to function like that of other VKD proteins (e.g., protein C, Esmon *et al.*, 1993) in appropriately positioning the molecule on the membrane, which facilitates Gas6 binding to its receptor. It should be noted that warfarin is currently used as an anticoagulant to treat millions of people, and the more recent appreciation of the breadth of VKD protein function has stimulated interest in how warfarin inhibition may impact other physiologies, for example, bone health and vascular calcification.

VKD carboxylation is also observed in nonmammalian metazoans. VKD carboxylase orthologs are present in fish, which have a hemostatic system (Jiang and Doolittle, 2003), as well as in organisms (*Conus*, *Urochordata*, and *Drosophila*) (Bandyopadhyay *et al.*, 2002; Czerwicz *et al.*, 2002; Kulman *et al.*, 2006; Li *et al.*, 2000; Walker *et al.*, 2001; Wang *et al.*, 2003) that lack a

clotting system and that evolved well before fish and mammals, indicating that Gla-containing proteins originally evolved for a function other than hemostasis. Snails of the *Conus* genus synthesize and eject small VKD peptides that paralyze their prey by antagonizing neurotransmission (Olivera *et al.*, 1985), and urochordates contain a putative tyrosine receptor kinase as well as other VKD proteins with unknown functions (Kulman *et al.*, 2006; Wang *et al.*, 2003). The role of carboxylation in *Drosophila* is unknown, as VKD proteins have not yet been revealed in this organism, and laboratory strains deleted in the carboxylase gene are viable (Bandyopadhyay *et al.*, 2006; Rong *et al.*, 2002). Until recently, the VKD carboxylase was thought to be restricted to the animal kingdom. VKD carboxylase orthologs had not been observed in fungi, plants, protozoa, or archaea bacteria despite the large number of sequenced genomes from these kingdoms. However, orthologs have now been detected in a small number of bacteria (Schultz, 2004), and an ortholog has been analyzed from the pathogen *Leptospira interrogans* that causes leptospirosis. The evolutionary analysis indicates that the VKD carboxylase was acquired by horizontal transfer during *L. interrogans* infection of a metazoan host (Rishavy *et al.*, 2005). As described below, the VKD ortholog appears to have been adapted for a role other than Glu carboxylation (Rishavy *et al.*, 2005).

II. VITAMIN K FORMS

Vitamin K comprises a family of forms (one phyloquinone and several menaquinones) that share the same methylated naphthoquinone nucleus but that differ in the isoprenyl side chain (Fig. 7.1A). Phyloquinone is found in green, leafy vegetables while menaquinones are present in animal products and in fermented foods (Booth and Suttie, 1998). Historically, long chain menaquinones generated by bacteria in the gut were thought to be an important source of vitamin K (Suttie, 1995); however, shorter chain dietary forms ($n = 6-8$, Fig. 7.1A) are now considered more physiologically relevant because of greater bioavailability. Interestingly, the menaquinone MK4 ($n = 3$, Fig. 7.1A), which is present in only small amounts in the diet, can be synthesized from phyloquinone in tissue (Davidson *et al.*, 1998; Ronden *et al.*, 1998), and MK4 is present in much higher amounts than phyloquinone in some organs (e.g., brain) (Thijssen and Drittij-Reijnders, 1996). Functional differences in phyloquinone and MK4 have been demonstrated by the ability of MK4, but not phyloquinone, to protect against arterial calcification in warfarin-treated rats (Spronk *et al.*, 2003), for reasons that remain to be established. The identification of a conversion pathway has raised the question of whether vitamin K has an additional function besides carboxylation, and proposed roles include involvement in sphingolipid biosynthesis (Carrié *et al.*, 2004), transcriptional regulation (Tabb *et al.*,

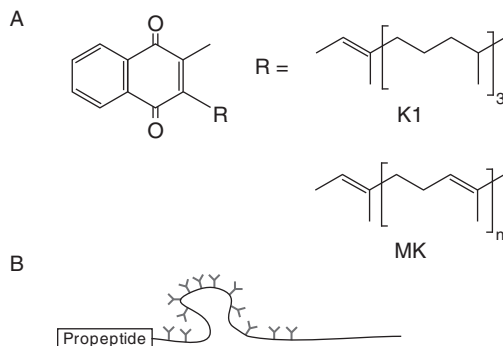


Figure 7.1 Structures of vitamin K and vitamin K-dependent (VKD) proteins. (A) Vitamin K is a family of forms that includes phyloquinone (K1) and the menaquinones (MK) which have a variable number of isoprenoid units. Dietary vitamin K is a quinone that requires reduction to the hydroquinone form for use by the carboxylase. (B) Most known VKD proteins contain an N-terminal propeptide and adjacent Gla domain that contains multiple carboxylated Glu residues (Glas, indicated by the Ys). The propeptide is proteolytically removed subsequent to carboxylation, and the Gla residues bind calcium and render VKD proteins active.

2003), and protection against neuronal cell death (Li *et al.*, 2003). Additional work will be required to determine whether these interesting possibilities are not manifestations of the diverse functions of VKD proteins.

III. MECHANISM OF CARBOXYLATION

The carboxylase uses the oxygenation of vitamin K to drive Glu carboxylation. Thus, the carboxylase is both an epoxidase that converts reduced vitamin K (KH_2 , Fig. 7.2) to vitamin K epoxide (KO) and a carboxylase that generates Gla from Glu. The carboxylation of Glu involves the formation of a carbanion intermediate which then attacks CO_2 to generate Gla (Suttie, 1985). The carbanion intermediate has a $\text{pK}_a \sim 25\text{--}28$, indicating that its formation requires a base far stronger than ones that could be provided by carboxylase residues. Chemical modeling studies indicated that the strong base might be a vitamin K intermediate (i.e., K^- in Fig. 7.2) with sufficient basicity to generate the carbanion (Dowd *et al.*, 1995). Generation of the strong base would require KH_2 deprotonation in order to react with O_2 , which led to the proposal that one function of the carboxylase is to provide an active site residue that acts as a weak base to deprotonate KH_2 . This mechanism, in which a weak carboxylase base generates a strong vitamin K base, was termed the “base amplification model” (Dowd *et al.*, 1995) (Fig. 7.2). As described in the following section, this weak base was recently identified as Lys218 (Rishavy *et al.*, 2006).

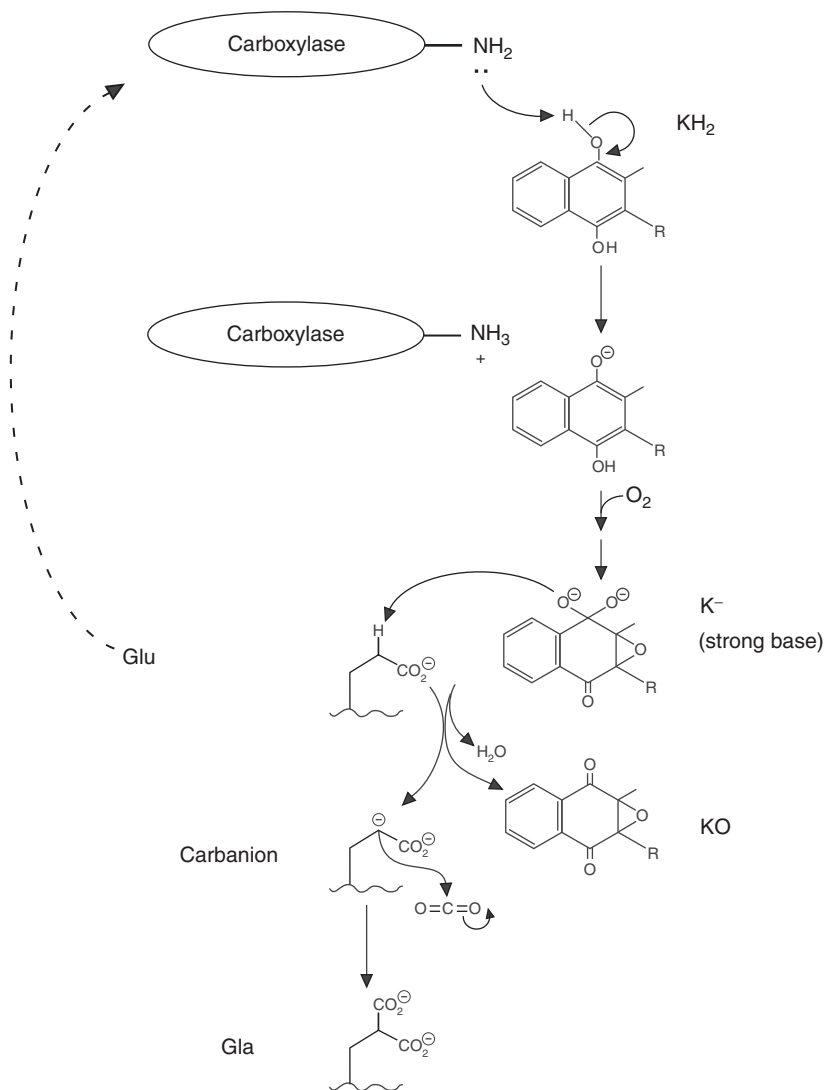


Figure 7.2 The carboxylase reaction. Vitamin K epoxidation is initiated when Lys218 in the carboxylase active site deprotonates reduced vitamin K (KH_2) to allow reaction with O_2 and the formation of a highly reactive strong base (e.g., the dialkoxide form shown, K^-). The strong base then deprotonates glutamyl residues (Glu) to generate a carbanion that reacts with CO_2 to form γ -carboxylated Glu (Gla), while the vitamin K base is protonated to form vitamin K epoxide (KO). Regulation of epoxidase activity by vitamin K-dependent protein (dashed arrow) (Sugiura *et al.*, 1997) ensures that vitamin K epoxidation does not occur when Glu substrate is not available.

The carboxylase selectively binds VKD proteins because they contain a specific sequence, which in most cases is a propeptide that is removed by proteolysis in the Golgi after the VKD proteins are carboxylated in the endoplasmic reticulum. The propeptide is usually positioned immediately upstream of the Gla domain that contains the Glus undergoing carboxylation (as in Fig. 7.1B). However, other domain organizations exist. For example, in matrix Gla protein the motif that mediates carboxylase binding is part of the mature protein and is straddled on both sides by the Gla domain (Price *et al.*, 1987). Likewise, *Conus* VKD peptides, which also contain both a Gla domain and sequence that binds the carboxylase, have been shown to contain either an N- or C-terminal carboxylase recognition sequence that is proteolytically removed after carboxylation (Brown *et al.*, 2005; Bush *et al.*, 1999). The *Conus* VKD peptides exhibit very little sequence homology with mammalian VKD proteins, and *Drosophila* VKD proteins are presumed to be dissimilar as well since orthologs have not been detected by homology searches. Nonetheless, interspecies reactivity occurs, as demonstrated by the *in vitro* carboxylation of mammalian VKD substrates by either *Conus* or *Drosophila* carboxylases (Bandyopadhyay *et al.*, 2006; Czerwicz *et al.*, 2002).

VKD protein binding to the carboxylase causes allosteric changes that regulate carboxylation. Propeptide binding increases carboxylase affinity for the Glu substrate (Knobloch and Suttie, 1987) and Glu binding modulates carboxylase affinity for propeptide (Presnell *et al.*, 2001). Binding of a substrate in which both domains are covalently attached increases the affinity of the carboxylase for the vitamin K cofactor (Soute *et al.*, 1992). VKD protein binding therefore results in more efficient utilization of vitamin K, which is significant because the availability of the reduced vitamin K cofactor limits carboxylation (discussed below). The carboxylase is also regulated so that vitamin K epoxidation does not occur in the absence of VKD protein binding (Sugiura *et al.*, 1997). This regulation is important in preventing highly reactive and undesirable vitamin K intermediates (i.e., K^- in Fig. 7.2) from being generated when Glus are not available for carboxylation.

Carboxylation occurs through a processive mechanism: multiple Glus (3–13 for individual VKD proteins) are converted to Glas in a single binding event in which the propeptide tethers the VKD proteins to the carboxylase throughout the multiple modifications (Morris *et al.*, 1995; Stenina *et al.*, 2001). Full carboxylation is required for activity and is normally observed but, as described below, can be disrupted when levels of vitamin K cofactor are low. Processive carboxylation reflects the relative rates of catalysis and release of VKD proteins from the carboxylase. *In vitro*, release of fully carboxylated VKD product is much slower than catalysis: single turnover studies with factor IX–carboxylase complexes show rapid carboxylation (minutes) but slow release (hours) (Hallgren *et al.*, 2002) and experiments with a substrate containing the propeptide and Gla domain show that release

of fully carboxylated product is ~ 50 -fold slower than catalysis (Lin *et al.*, 2004). However, these relative rates are different in cells, as described in more detail below.

The carboxylase binds VKD propeptides with different affinities (Stanley *et al.*, 1999b). The VKD propeptide usually contains three highly conserved residues (at positions -6 , -10 and -16 relative to the start of the mature proteins), and mutational analyses of these residues, as well as others within the propeptide, indicates their importance in modulating carboxylase binding (Furie *et al.*, 1999; Stanley *et al.*, 1999a). The osteocalcin propeptide is notable in lacking two of the three conserved residues (at -6 and -10) and in exhibiting weak association with the carboxylase. For example, the affinity of the osteocalcin propeptide is at least 10^4 -fold lower than that of the factor X propeptide in a competition assay that measures propeptide inhibition of carboxylation (Stanley *et al.*, 1999b). However, mature osteocalcin (i.e., that lacks propeptide) is also unusual in that it is bound by the carboxylase with high affinity (Engelke *et al.*, 1991; Houben *et al.*, 1999). These data implicate two sites of osteocalcin interaction with the carboxylase, one that is low affinity (via the propeptide) and one that is high affinity (via a sequence not yet identified). Two sites of interaction could potentially increase the efficiency of the reaction by appropriately positioning the Gla domain within the carboxylase active site to facilitate processive carboxylation of the multiple Glus. The observations with osteocalcin therefore raise the question of whether all mammalian VKD proteins interact with the carboxylase at two sites. Even the related hemostatic VKD propeptides show a wide range of interaction with the carboxylase, for example, with 100-fold weaker binding of the prothrombin propeptide than factor X propeptide in the competition assay described above. The carboxylase has a low-affinity site of interaction (at residues 343–360, Fig. 7.3) with hemostatic VKD proteins that lack propeptide (Pudota *et al.*, 2001), suggesting that two sites of interaction is a universal feature governing reactivity of carboxylase with VKD proteins.

IV. FUNCTIONAL REGIONS OF THE CARBOXYLASE

Only a limited number of functional carboxylase residues are known as the carboxylase is a large integral membrane protein without homology to other known proteins. A 2D crystal structure has been reported which indicates a monomeric structure but not functional amino acids (Schmidt-Krey *et al.*, 2007). The identification of naturally occurring missense mutations has been particularly informative in guiding structure–function relationships. The human carboxylase gene is autosomal (Wu *et al.*, 1997) and until recently, only five mutations were known (Fig. 7.3), four of which

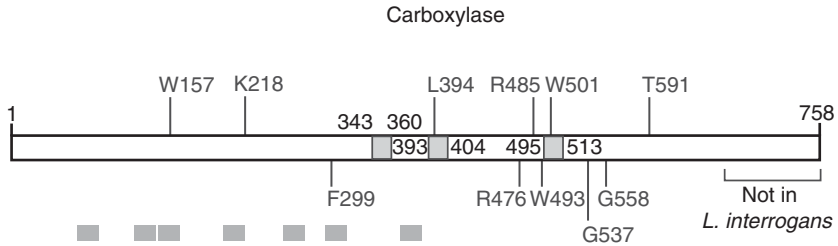


Figure 7.3 Functional regions of the human vitamin K-dependent (VKD) carboxylase. Residues shown above the box include the catalytic base (Lys218) that initiates carboxylation and five naturally occurring carboxylase mutations that cause severe bleeding due to combined VKD protein deficiency. The naturally occurring mutations shown below the box were identified in patients with a phenotype similar to pseudoxanthoma elasticum. The shaded regions within the box represent, from left to right, a sequence that binds mature VKD proteins (residues 343–360), the Glu-binding domain (residues 393–404) and the propeptide-binding domain (residues 495–513). The grey bars and bracket below the box indicate potential membrane spanning sequences and C-terminal sequences that are not present in the *L. interrogans* VKD ortholog, respectively.

have been tested *in vitro* (Darghouth *et al.*, 2006; Mutucumarana *et al.*, 2000; Soute *et al.*, 2004). All of these mutations cause serious bleeding complications due to combined VKD hemostatic factor deficiency, consistent with there being only one VKD carboxylase responsible for modifying all VKD proteins. Remarkably, several additional carboxylase missense mutations were recently identified in patients with a phenotype similar to pseudoxanthoma elasticum PXE (Vanakker *et al.*, 2007). Classic PXE results from defects in an ATP-dependent transmembrane transporter, and a hallmark of this disease is excessive skin folding. Patients with the carboxylase mutations share this phenotype but differ in that they also exhibit impaired hemostatic VKD protein activity. A notable point is that the carboxylase defect does not appear to be life-threatening as was the case with the previously known carboxylase mutations, since the patients with the PXE-like phenotype presented because of skin abnormalities rather than bleeding complications. Calcification of elastic fibers in the dermis is a characteristic of PXE, raising the question of whether the carboxylase mutations have more of an impact on the carboxylation of nonhemostatic VKD proteins (e.g., matrix Gla protein) than the hemostatic VKD proteins.

The first mutant identified as causing combined deficiency of hemostatic VKD proteins was L394R (Fig. 7.3) (Brenner *et al.*, 1998). Leu394 resides within a highly evolutionarily conserved region (Begley *et al.*, 2000), and substitutions in Leu394 and in the neighboring residues Tyr395 and Trp399 cause large decreases in Glu binding (Mutucumarana *et al.*, 2000, 2003), implicating this region as important to Glu interaction. Two of the mutants (i.e., L394R and W399A) also show decreased propeptide binding (Mutucumarana *et al.*, 2003), consistent with the interrelationship between

Glu and propeptide binding described in the previous section. However, decreased Glu, but not propeptide, binding is observed with Y395A, supporting the role of this region to Glu binding. Leu394, Tyr395, and Trp399 are all hydrophobic residues, indicating that the carboxylase is not binding Glus through ionic interaction and may instead be binding either the peptide backbone or hydrophobic residues that are adjacent to the Glus and are located throughout the Gla domain.

Trp501, whose substitution by Ser also causes combined VKD protein deficiency (Spronk *et al.*, 2000), appears to be part of the propeptide-binding site. This amino acid resides within a region that was originally proposed to be a regulatory sequence because of its homology to matrix Gla protein (Price and Williamson, 1993). Thus, binding of this carboxylase sequence to the carboxylase propeptide-binding site was suggested to maintain the carboxylase in an inactive state in the absence of propeptide-containing VKD substrate (Price and Williamson, 1993). Kinetic analysis of W501S (Soute *et al.*, 2004) was consistent with this role but could also be explained by this residue instead being part of the actual propeptide-binding site. Subsequent analysis of neighboring residues used fluorescence anisotropy as an additional approach to distinguish these two roles in studies that support the region around Trp501 being the propeptide-binding site rather than an internal regulatory sequence (Lin *et al.*, 2002).

Naturally occurring mutations in residues Trp157 and Thr591, which are both evolutionarily conserved, have also been tested *in vitro*. The W157R and T591N mutations were identified in a patient who also contained a third carboxylase missense mutation (D31N), and analysis of enzymes bearing the individual substitutions indicated that compound heterozygosity in W157R and T591N could account for combined VKD protein deficiency (Darghouth *et al.*, 2006). The patient did not respond to vitamin K supplementation, which contrasts the response of patients with the L394R or W501S mutations who showed at least partial restoration of VKD function when supplemented with vitamin K (Brenner *et al.*, 1998; Spronk *et al.*, 2000). Their response to vitamin K is consistent with the increased carboxylase affinity for vitamin K effected by VKD protein binding discussed in the previous section. Thus, decreased propeptide binding in W501S or Glu binding in L394R would impair vitamin K binding, explaining why vitamin K supplementation improves carboxylation in these patients. The lack of response in the patient with the W157R and T591N mutations therefore suggests that Trp157 and Thr591 have roles other than VKD protein binding.

Comparison of evolutionarily distinct VKD carboxylase orthologs has also been informative in suggesting functional regions, and the *L. interrogans* VKD ortholog (Rishavy *et al.*, 2005) has been particularly valuable because its structure is more divergent. Interestingly, the *L. interrogans* VKD ortholog differs in several ways from the mammalian carboxylases: it exhibits

vitamin K epoxidation but not detectable Glu carboxylation, it is divergent in the Glu-binding region (Fig. 7.3), and it shows unregulated vitamin K epoxidation, that is, it occurs in the absence of Glu substrate (Rishavy *et al.*, 2005). These properties suggest that *L. interrogans* has adapted the VKD ortholog for some function other than Glu carboxylation, such as using vitamin K epoxidation to drive a new reaction or causing oxidative damage in the infected organism or depleting circulating vitamin K and consequently inhibiting VKD protein carboxylation in the host. The ability of the *L. interrogans* VKD ortholog to perform vitamin K epoxidation is of interest with regard to carboxylase residues required for this function. Previous studies showing reduced vitamin K epoxidase activity in a truncated bovine carboxylase lacking the C-terminal 82 amino acids suggested that the C-terminus comprises at least part of the vitamin K epoxidation domain (Roth *et al.*, 1995). However, these sequences are not even present in the *L. interrogans* VKD ortholog (Fig. 7.3) (Rishavy *et al.*, 2005), indicating that there must be some other explanation for the reduced activity in the truncated bovine carboxylase.

The evolutionary divergence of the *L. interrogans* VKD ortholog was also useful in identifying the catalytic base that initiates the carboxylation reaction. This residue was initially thought to be a cysteine based on inhibitor studies with thiol-specific reagents like *N*-ethylmaleimide (NEM) (Canfield, 1987; Friedman and Shia, 1976; Mack *et al.*, 1976; Suttie *et al.*, 1979), and biochemical mapping with [¹⁴C]-NEM revealed Cys99 and Cys450 as NEM-reactive residues (Pudota *et al.*, 2000). However, a cysteine-less carboxylase mutant with all 10 cysteine residues substituted by alanine was subsequently shown to be active (Rishavy *et al.*, 2004), indicating that cysteine is not the catalytic base. The unusual NEM reactivity of this mutant implicated an amine, and a comparison of the *L. interrogans* VKD ortholog with metazoan VKD carboxylases narrowed the number of candidate residues to four, only one (Lys218) of which resulted in complete loss of activity when substituted by alanine (Rishavy *et al.*, 2006). The activity of this K218A mutant was restored by exogenously added amines and activity rescue depended upon the base strength of added amine (i.e., the basicity of the amine) (Rishavy *et al.*, 2006), indicating that Lys218 is the catalytic base that deprotonates vitamin K to initiate epoxidation and consequent carboxylation (Fig. 7.2).

The location of Lys218 is of interest with regard to how vitamin K accesses the carboxylase active site. Thus, vitamin K and Glu substrate must be in close proximity for reactivity; however, vitamin K is in the membrane while the VKD Gla domain and carboxylase Glu-binding site are in the lumen of the endoplasmic reticulum (Carlisle and Suttie, 1980). How the vitamin K and Glu substrate are juxtaposed is unknown but is likely impacted by the topology of the carboxylase in the membrane. The carboxylase cDNA predicts seven membrane-spanning sequences (Fig. 7.3;

Wu *et al.*, 1991), five of which cause membrane insertion in an *in vitro* assay (Tie *et al.*, 2000); however, as previously discussed (Berkner, 2005), some of the sequences may instead be monotopic, that is, inserting into only one leaflet of the membrane. This motif occurs in other enzymes in the endoplasmic reticulum which have luminal or cytoplasmic active sites but access a hydrophobic substrate from the membrane (Bracey *et al.*, 2004). The monotopic sequences facilitate access by forming a tight junction between membrane and aqueous environment, allowing hydrophobic substrates to react in the active sites. It is interesting to note that residues 197–217 in the carboxylase were predicted to be membrane-spanning sequences but did not result in membrane insertion *in vitro* (Tie *et al.*, 2000). These residues, then, may comprise a monotopic sequence, which would position Lys218 appropriately for interacting with both vitamin K in the membrane and with the luminal active site.

As discussed in the previous section, vitamin K epoxidation is regulated by the Glu substrate, and the identification of Lys218 as the catalytic base raises an interesting possibility for the mechanism of regulation. Lysine residues are normally protonated at physiological pH, but Lys218 must be unprotonated to function as the catalytic base, indicating that the carboxylase somehow maintains it in an unprotonated state. One mechanism used by other enzymes is the presence of a second lysine, where close juxtapositioning of two positively charged residues decreases the affinity of one lysine for a proton and results in deprotonation at physiological pH. This mechanism in the carboxylase is attractive in that it could explain Glu substrate regulation of vitamin K epoxidation: interaction of a negatively charged Glu with the modulating lysine would neutralize its charge and restore the inherent basicity of Lys218, allowing this base to deprotonate vitamin K to initiate the reaction (Fig. 7.2). Lys218 would therefore be functional for vitamin K epoxidation only when Glu substrate is present.

V. VITAMIN K REDUCTION IS REQUIRED FOR CARBOXYLATION IN TISSUE

The vitamin K epoxide (KO) product of carboxylation is continuously recycled to the reduced vitamin K (KH₂) cofactor by VKOR (Fig. 7.4), which can explain why Glu to Gla conversion is so much higher than dietary vitamin K intake (Suttie, 1985). VKOR, whose activity was identified soon after the discovery of the carboxylase (Suttie, 1985), is the target of anticoagulant therapies with drugs like warfarin (Hirsh *et al.*, 2001). Inhibition by warfarin blocks the supply of KH₂ and leads to the secretion of poorly carboxylated VKD proteins with impaired biological activities. Combined VKD protein deficiency can also result from mutations in

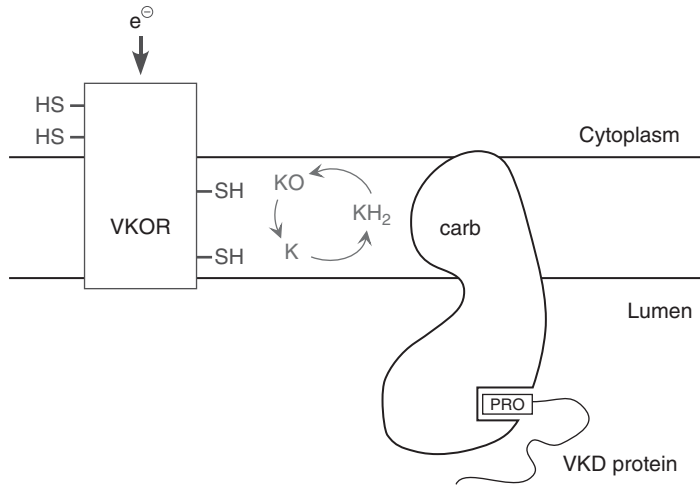


Figure 7.4 Vitamin K recycling. Vitamin K-epoxide reductase (VKOR) uses active site membrane-localized thiols to reduce vitamin K epoxide (KO) to an intermediate quinone (K) and then to vitamin K hydroquinone (KH₂), the cofactor for the carboxylase (carb). The active site thiols become oxidized during KO reduction, inactivating VKOR, and activity regeneration is accomplished by an unidentified redox protein that provides electrons (e⁻) to VKOR. Vitamin K in the diet exists in the quinone form, but most KH₂ comes from KO recycling by VKOR. At least one tissue (liver) also contains a quinone reductase able to convert K (but not KO) to KH₂. This reductase is relatively insensitive to warfarin and provides an antidotal pathway for generating KH₂ when bleeding complications arise during warfarin therapy.

VKOR, and one such mutant (R98W) has been identified (Rost *et al.*, 2004a), which was instrumental in identifying the gene for VKOR. Thus, linkage analysis of R98W, as well as mutations causing warfarin resistance, localized VKOR to a ~4 Mb region on chromosome 16 (Fregin *et al.*, 2002), and VKOR identification within this region was subsequently accomplished by using either sequence analysis or siRNA knockdown (Li *et al.*, 2004; Rost *et al.*, 2004a). The gene encoded a surprisingly small enzyme of 163 amino acids that could reduce KO and that was inhibitable by warfarin. Algorithms predict an integral membrane protein with three or four transmembrane sequences, and three have been indicated by functional tests (Tie *et al.*, 2005). Identification of the gene revealed homologues in a diverse range of organisms (e.g., plants and archaea) that contain vitamin K forms (Fig. 7.1A) but do not contain the VKD carboxylase (Goodstadt and Ponting, 2004). The functional role for VKOR in these organisms is not yet known.

Early biochemical studies with thiol-specific inhibitors suggested the importance of cysteines to VKOR activity (Fasco *et al.*, 1983; Whitlon *et al.*, 1978), and VKOR contains four evolutionarily conserved cysteine residues (Goodstadt and Ponting, 2004). Mutational analysis implicates a

functional role for all four of these amino acids (Rost *et al.*, 2005). Two cysteines are part of a CXXC motif known to be involved in redox chemistry in other enzymes, and topological mapping indicates their location in the membrane (Tie *et al.*, 2005), like vitamin K (Fig. 7.4), making it likely that they function in reducing KO. The role of the remaining two cysteines is unknown. Oxidation of the active site thiols during KO reduction inactivates VKOR, and activity regeneration requires a redox protein whose identity has not yet been established. Several candidate proteins have been shown to stimulate VKOR activity *in vitro* (e.g., Silverman and Nandi, 1988; Thijssen *et al.*, 1994); however, even a bacterial enzyme is functional in these assays and so the physiologic relevance of these redox proteins to VKOR function in carboxylation is unknown.

Other residues implicated in VKOR function include Arg 98, which as described above, causes combined VKD protein deficiency when substituted by Trp (Rost *et al.*, 2004a). A functional role for residue 57 is also indicated by the conservation of a Ser or Thr at this position in all VKOR homologues (Goodstadt and Ponting, 2004), and substitution by Ala eliminates activity (Rost *et al.*, 2005). The only other residues known to be of functional importance are those whose substitution causes warfarin resistance (Harrington *et al.*, 2005; Rost *et al.*, 2004a). The *in vivo* mechanism(s) of these mutations remain to be determined as these mutants exhibit warfarin sensitivity, rather than resistance, when tested *in vitro* (Rost *et al.*, 2004a).

VI. CARBOXYLATION INTERFACES WITH SECRETION

Carboxylation occurs during VKD protein secretion, and some of the steps in carboxylation are almost certainly mediated by secretory components. For example, association of VKD proteins with the carboxylase (Fig. 7.5) likely involves chaperones, which have been implicated in the assembly of other multicomponent complexes in the endoplasmic reticulum (Hammond and Helenius, 1995). Multiple VKD proteins engage the carboxylase in many tissues (e.g., liver and bone). Whether their association with the carboxylase depends solely on the amount of VKD protein synthesized or whether there is a distinction due to one or several chaperones promoting association is unknown. Once bound, the VKD proteins are multiply carboxylated before release and, as discussed in the following section, the balance between rate of catalysis and release is critical in generating a fully carboxylated VKD product. Released VKD proteins then exit the endoplasmic reticulum and transit through the Golgi where they undergo additional posttranslational modifications (e.g., propeptide processing and glycosylation) as they are secreted to the cell surface or out of the cell. Intracellular analysis in cultured cells indicates that endoplasmic

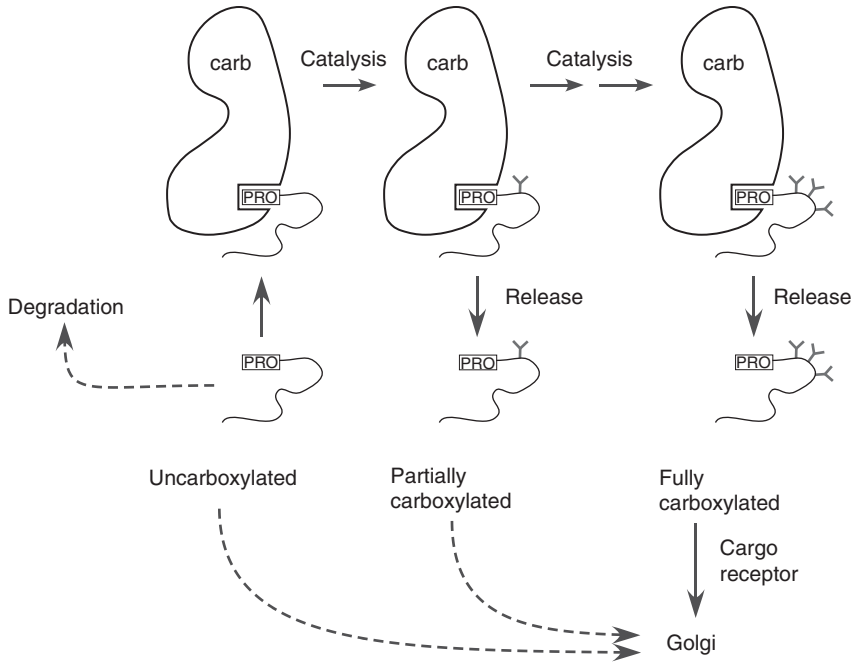


Figure 7.5 Carboxylation of vitamin K dependent (VKD) proteins in the endoplasmic reticulum. Most VKD proteins contain a propeptide (PRO) that tethers them to the carboxylase (carb) throughout multiple carboxylations (indicated by Y). Normally, the VKD proteins are fully carboxylated, released, and then exit the endoplasmic reticulum, most likely via cargo receptors that have yet to be identified. As discussed in the text, abnormal conditions can result in the buildup of uncarboxylated VKD proteins and to the release of partially carboxylated VKD intermediates, which are either secreted or degraded.

reticulum egress is unusually slow, which may allow more time for carboxylation to occur and which contrasts the observed rapid transit of VKD proteins through the Golgi (Hallgren *et al.*, 2006). The mechanism by which VKD proteins exit the endoplasmic reticulum is unknown. Cargo receptors, several of which have been shown to mediate this step with other proteins, have not been identified for VKD proteins, and consequently it is not known whether all VKD proteins engage the same cargo receptor(s), or what signals trigger cargo receptor recognition.

Some uncarboxylated VKD proteins also have an alternative fate, which is degradation (Fig. 7.5). Substantially less protein C and protein Z are secreted in the absence than in the presence of vitamin K (Souri *et al.*, 2005; Tokunaga *et al.*, 1995), and in the case of protein C, this difference has been attributed to preferential degradation of poorly carboxylated protein C via the proteasomal pathway (Tokunaga *et al.*, 1995). This filtering mechanism can therefore decrease the amount of uncarboxylated and

nonfunctional VKD protein that is secreted. However, not all VKD proteins show this form of regulation. Analysis by pulse-chase in cells lacking vitamin K shows that all newly synthesized factor IX is ultimately secreted (Hallgren *et al.*, 2002, 2006), which indicates that degradation of uncarboxylated protein does not occur with this VKD protein. Thus, the secretory process does not necessarily have the same impact on all VKD proteins.

VII. THE CAPACITY OF VITAMIN K-DEPENDENT PROTEIN CARBOXYLATION IS LIMITED IN CULTURED CELLS

Efficient carboxylation is limited in mammalian cells. Low-level expression results in the secretion of fully carboxylated VKD proteins. However, high-level expression saturates the capacity for carboxylation because the carboxylase is not sufficiently rapid in turning over the larger amounts of VKD protein synthesized, and so uncarboxylated VKD protein is secreted along with fully carboxylated protein. This limitation in carboxylation has been a major impediment for producing VKD proteins for therapeutic applications, for example, factor VII and factor IX in the treatment of hemophilia and protein C in the protection against sepsis (Fourrier, 2004; Hedner, 2001; Roberts *et al.*, 2004; White *et al.*, 1998). Carboxylase overexpression does not improve carboxylation (Hallgren *et al.*, 2002; Rehemtulla *et al.*, 1993) even though the recombinant carboxylase is functional, that is, resulting in a proportional increase in both activity and number of productive factor IX-carboxylase complexes to the amount of carboxylase protein overexpressed (Hallgren *et al.*, 2002). Those studies suggested that r-carboxylase cannot increase carboxylation because the supply of reduced vitamin K cofactor is limiting (Hallgren *et al.*, 2002). Subsequent studies showed that overexpression of VKOR and consequent increase in the reduced vitamin K cofactor resulted in increased VKD protein carboxylation (Hallgren *et al.*, 2006; Sun *et al.*, 2005; Wajih *et al.*, 2005). Interestingly, however, the effect of r-VKOR was small (~ 2 -fold) and considerably less than the amount of VKOR overexpressed. In contrast to the results in cells, proportional increases in factor IX carboxylation were observed *in vitro* where DTT substitutes for the redox protein that regenerates VKOR activity (Hallgren *et al.*, 2006). These data suggest that the reason why r-VKOR only causes a small effect in cells is because VKOR overexpression saturates the capacity of the redox protein that regenerates VKOR activity. A functional test of the redox protein, once it is identified, will clearly be of interest.

The increase in carboxylation with VKOR overexpression was somewhat surprising and is of interest with regard to how fully carboxylated VKD proteins are generated. As described in an earlier section, the release of

fully carboxylated VKD proteins is much slower than catalysis during *in vitro* carboxylation, and a slow rate of release has been proposed to account for processive carboxylation. However, catalysis must be rate-limiting in cells for there to be an increase in carboxylation with VKOR overexpression. The difference in catalytic rates in cells versus *in vitro* is presumably due to the limiting amount of reduced vitamin K generated by VKOR in cells versus the saturating amounts of chemically reduced vitamin K used *in vitro*. Nonetheless, cells generate fully carboxylated VKD proteins, which means that even though the rate of catalysis is slower than the rate of release of fully carboxylated VKD proteins, it must still be faster than the release of partially carboxylated intermediates. The carboxylase must therefore be able to distinguish fully carboxylated product from partially carboxylated intermediates, which both contain the propeptide that mediates VKD protein binding to the carboxylase. One difference between VKD intermediates and product is the presence or absence of Glu residues, and studies with Glu or Gla containing substrates show that loss of Glu-binding increases release (Lin *et al.*, 2004). However, the effect is small (Lin *et al.*, 2004), and whether larger differences exist *in vivo* is unknown. Larger differences in release are suggested by the fact that cells producing low-levels of VKD protein generate only fully carboxylated VKD proteins and by the observation that even a prothrombin mutant that is deleted in the Gla domain and has the propeptide joined to an unrelated Glu-rich region of the molecule is multiply carboxylated (Furie *et al.*, 1997).

Warfarin inhibition of VKOR decreases the supply of reduced vitamin K cofactor and therefore the rate of catalysis. The slower turnover of VKD proteins by the carboxylase results in a buildup of uncarboxylated proteins, because synthesis of VKD proteins still occurs, and the uncarboxylated proteins can be secreted into blood. Partially carboxylated forms of at least one VKD protein (prothrombin, which is the only one analyzed) are also secreted (Liska and Suttie, 1988; Malhotra, 1972), indicating that the catalytic rate is now slower than the rate of release of partially carboxylated forms (Fig. 7.5). The goal of warfarin therapy is to dampen rather than abolish carboxylation, and the therapeutic range of VKD protein activity is ~30–40%. Curiously, the activities of all hemostatic VKD proteins fall to this level, despite the fact that there is as much as a 100-fold difference in the affinity of the carboxylase for the various hemostatic VKD propeptides (discussed in an earlier section). A related observation is the response to warfarin of hemophiliacs with mutations in the factor IX propeptide (at positions -9 and -10) (Chu *et al.*, 1996; Oldenburg *et al.*, 1997; Stanley *et al.*, 1999a). These patients exhibit normal hemostasis until administered warfarin, which results in a selective decrease in factor IX activity to $\leq 1\%$, well below the activity levels of the other hemostatic VKD proteins. Functional analysis indicated decreased affinity of the carboxylase for the mutated propeptides (Stanley *et al.*, 1999a), which became significant only

when the rate of catalysis was slowed by warfarin. A puzzling aspect, however, is that the mutant propeptide substituted at the -9 position has a carboxylase affinity similar to that of the propeptides of prothrombin and protein C, yet these two proteins are only decreased to the 30–40% therapeutic range (Lind *et al.*, 1997). The combined observations indicate, then, that release of VKD proteins from the carboxylase is not dictated solely by the propeptide and that other factors may impact release, for example, a second site of VKD protein–carboxylase interaction or secretory components that facilitate the process of carboxylation.

The issue of whether secretory components impact carboxylation is also of interest with regard to the rates of VKD protein turnover that are observed *in vitro* and in cells. *In vitro*, release of propeptide-containing fully carboxylated protein takes ~ 45 min (Lin *et al.*, 2004), which is similar to the amount of time required for the turnover of a single factor IX molecule (i.e., that undergoes 12 Glu to Gla conversions) (Morris *et al.*, 1995; Stenina *et al.*, 2001). In cells, the rate of catalysis must be at least twofold slower than rate of release because catalysis is rate limiting in cells and can be increased twofold by VKOR overexpression (discussed above). Therefore, if the rate of release is the same in cells as *in vitro*, VKD protein turnover would take at least 135 min. This value is substantially slower than the 4-min turnover rate determined in cells (Hallgren *et al.*, 2006), indicating that release is much faster in cells than *in vitro*. Removal of carboxylated VKD product by secretion could shift reaction equilibrium but does not appear to be the reason for faster release, at least in cultured cells, because exit of carboxylated VKD protein from the endoplasmic reticulum is slow (Hallgren *et al.*, 2006). The difference in rate of release of fully carboxylated VKD proteins *in vitro* versus in cells thus raises the question of whether this step is facilitated by cellular components.

VIII. SUMMARY

Recent advances have revealed considerable insight in understanding VKD protein carboxylation while also raising new questions regarding this complex process. Key questions in defining VKOR function are the identity of the redox protein that regenerates VKOR activity, the mechanisms for KO reduction and warfarin inhibition, and how VKOR interacts with the carboxylase to provide the KH_2 cofactor. Important questions regarding the carboxylase include defining how vitamin K epoxidation is regulated by VKD protein binding, determining how processive carboxylation is accomplished and whether the efficiency of processivity is the same for all VKD proteins, and uncovering the reason why carboxylase mutations cause a PXE-like phenotype. Obtaining structural information on the carboxylase

and VKOR, and determining whether secretory proteins impact the function of carboxylation are also essential goals. Finally, studies on VKD proteins with unknown functions (e.g., the transmembrane Gla proteins in humans or VKD proteins in invertebrates) will be of interest in defining the full breadth of physiologies impacted by vitamin K.

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VITAMIN K-DEPENDENT γ -GLUTAMYL CARBOXYLATION: AN ANCIENT POSTTRANSLATIONAL MODIFICATION

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Abstract

The vitamin K-dependent carboxylase carries out the posttranslational modification of specific glutamate residues in proteins to γ -carboxy glutamic acid (Gla) in the presence of reduced vitamin K, molecular oxygen, and carbon dioxide. In the process, reduced vitamin K is converted to vitamin K epoxide, which is

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subsequently reduced to vitamin K, by vitamin K epoxide reductase (VKOR) for use in the carboxylation reaction. The modification has a wide range of physiological implications, including hemostasis, bone calcification, and signal transduction. The enzyme interacts with a high affinity γ -carboxylation recognition sequence (γ -CRS) of the substrate and carries out multiple modifications of the substrate before the product is released. This mechanism ensures complete carboxylation of the Gla domain of the coagulation factors, which is essential for their biological activity. γ -Carboxylation, originally discovered in mammals, is widely distributed in the animal kingdom. It has been characterized in sea squirt (*Ciona intestinalis*), in flies (*Drosophila melanogaster*), and in marine snails (*Conus textile*), none of which have a blood coagulation system similar to mammals. The cone snails express a large array of γ -carboxylated peptides that modulate the activity of ion channels. These findings have led to the suggestion that γ -carboxylation is an extracellular posttranslational modification that antedates the divergence of molluscs, arthropods, and chordates. I will first summarize recent understanding of γ -carboxylase and γ -carboxylation gleaned from experiments using the mammalian enzyme, and then I will briefly describe the available information on γ -carboxylation in *D. melanogaster* and *C. textile*. © 2008 Elsevier Inc.

I. INTRODUCTION

In the course of his studies on cholesterol metabolism, Dam (1935) discovered vitamin K (Koagulations vitamin) and observed that its deficiency was associated with hemorrhagic disorder. Subsequent investigation into hemorrhagic disease in cattle following ingestion of spoiled clover led to the identification of a vitamin K antagonist bishydroxycoumarin (dicoumarol). Vitamin K acts as a cofactor in the posttranslational modification of certain glutamic acid residues in proteins to γ -carboxy glutamic acid (Gla) (Nelstuen *et al.*, 1974; Stenflo *et al.*, 1974). These proteins, referred to as vitamin K-dependent (VKD) proteins, play roles in blood coagulation. Later VKD proteins involved in bone metabolism were characterized. Factors VII, IX, X, prothrombin, protein C, protein S, and protein Z as well as the bone proteins matrix Gla protein and bone Gla proteins were the VKD proteins initially characterized. Other Gla-containing proteins that have been characterized include Gas6, a protein homologous to the anti-coagulation factor S (Manfioletti *et al.*, 1993), proline-rich Gla proteins 1 and 2 (PRGP1 and PRGP2) (Kulman *et al.*, 1997), and transmembrane Gla proteins 3 and 4 (TMG3 and TMG4) (Kulman *et al.*, 2001).

Gla-domain proteins have recently been described in the *urochordates*, tunicates and sea squirt (Jiang and Doolittle, 2003; Kulman *et al.*, 2006; Wang *et al.*, 2003).

A distinct class of γ -carboxylated molecules was described in 1984 (McIntosh *et al.*, 1984). The unexpected discovery of Gla in 1984 in the

venom of the invertebrate marine cone snails (*Conus*) (McIntosh *et al.*, 1984) introduced a new perspective to VKD proteins—namely, the evolutionary antiquity of the molecule and the possibility of its universal occurrence in novel biological molecules and physiological contexts. A multitude of Gla-containing peptides from cone snails have since been characterized. As much as 5% of the amino acids in *Conus* venom peptides have been estimated to be Gla (Hauschka *et al.*, 1988).

An ortholog of the γ -carboxylase with epoxidase but no carboxylase activity has been identified in the bacterial pathogen *Leptospira interrogans* (serovars Lai and Copenhageni) (Rishavy *et al.*, 2005).

Gla residues are essential for the activity of VKD proteins. They confer upon these proteins the ability to bind Ca^{++} , which result in conformational changes essential for their interaction with phospholipids in membranes (Furie and Furie, 1979; Nelsestuen, 1976; Nelsestuen *et al.*, 1974; Stenflo, 1999; Stenflo *et al.*, 1974).

Inhibition of posttranslational γ -carboxylation of coagulation factors results in hemorrhagic disease, while deficiency in matrix Gla protein results in improper calcification of arteries and cartilage in mice, resulting in short stature, osteopenia, and fractures (Luo *et al.*, 1997). A higher level of vitamin K is necessary to maintain bone mineralization compared to that for hemostasis (Vermeer and Hamulyak, 2004). Vitamin K is essential for frontonasal and metaphyseal ossification (Menger *et al.*, 1997; Van Kien *et al.*, 1998), and vitamin K deficiency in pregnant mothers due to malabsorption or warfarin treatment results in embryopathy. Vitamin K supplementation is recommended to prevent embryopathy especially at early stages of pregnancy when vitamin K deficiency is teratogenic.



II. REVIEWS

Mechanistic studies on γ -carboxylation have been carried out using mammalian γ -carboxylases, and the excellent reviews available on the subject should be consulted for comprehensive views on the subject (Berkner, 2005; Berkner and Runge, 2004; Furie *et al.*, 2003; Presnell and Stafford, 2002; Stafford, 2005; Stenflo and Dahlbäck 2001; Vermeer, 1990; Wu *et al.*, 1997c).



III. γ -CARBOXYLATION REACTION

The posttranslational modification of glutamate residues to γ -carboxyglutamate is carried out by a microsomal enzyme, VKD γ -glutamyl carboxylase (GGCX), in the presence of reduced vitamin K (KH_2), CO_2 ,

and O_2 (Fig. 8.1). In the process, KH_2 is converted to vitamin K epoxide (KO). Gla is not reused in protein synthesis and is excreted in urine. The reduction of KO to K by vitamin K epoxide reductase (VKOR) and subsequently to KH_2 either by the same enzyme or by a different reductase is necessary to supplement the dietary intake of vitamin K to meet the requirements for Gla synthesis. The stoichiometry of KH_2 used, KO formed, and Gla synthesized is equimolar.

Vitamin K is a group of compounds that are 2-methyl-1,4-naphthaquinone derivatives: phyloquinone, or vitamin K1, is a 3-phytyl derivative; menaquinone, or vitamin K2, is a $(3\text{-isoprene})_n$ derivative; and menadione, or vitamin K3, lacks substitutions at the 3 position.

γ -Carboxylase activity was first reported by Esmon *et al.* (1975), and the enzyme was subsequently cloned and purified by Wu *et al.* (1991a,b) and Berkner *et al.* (1992). The enzyme contains 758-amino acid residues. The gene is about 13 kb long and contains 15 exons (Wu *et al.*, 1997b) and has been mapped to p12 of chromosome 2 (Kuo *et al.*, 1995).

Genes encoding γ -carboxylase have been cloned from the cone snail *Conus textile* (Bandyopadhyay *et al.*, 2002; Czerwiec *et al.*, 2002), fly *Drosophila melanogaster* (Li *et al.*, 2000; Walker *et al.*, 2001), and sea squirt *Ciona intestinalis* (Kulman *et al.*, 2006).

A. Processivity of catalysis

Blood coagulation VKD proteins containing between 9 and 13 Gla residues need to be completely modified for biological function. Undercarboxylation of the proteins leads to severe impairment of biological activity

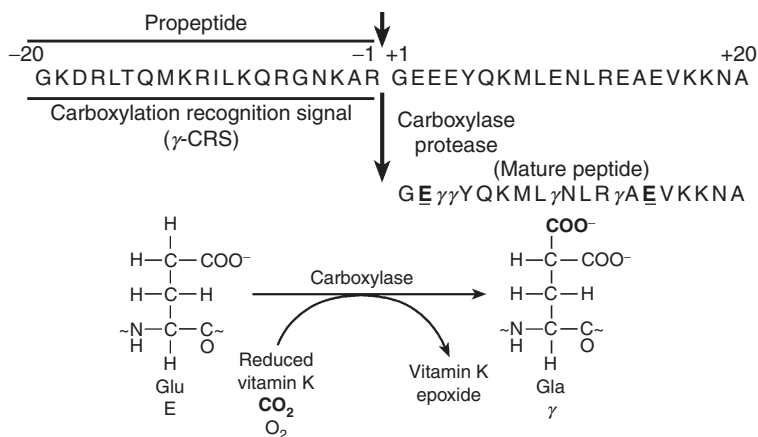


Figure 8.1 Schematic of reaction catalyzed by γ -glutamyl carboxylase. γ -Carboxylation of a molluscan (*Conus*) substrate, conantokin-T, is shown. The specificity of the enzyme is underscored by the fact that Glu² and Glu¹⁶ are not carboxylated.

(Martinez *et al.*, 2001). Complete carboxylation of a molecule is achieved by processivity of the enzyme in which it is tethered to the substrate at the γ -carboxylation recognition sequence (γ -CRS), and the rate of carboxylation is significantly higher than substrate release (Morris *et al.*, 1995; Stenina *et al.*, 2001).

IV. VITAMIN K CYCLE

A. Epoxide reductase

Vitamin K 2,3 epoxide is reduced to vitamin K and subsequently to the hydroquinone, which then participates in the γ -carboxylation reaction (Fig. 8.2). Li *et al.* (2004) and Rost *et al.* (2004a) have recently characterized VKOR, the enzyme responsible for the reduction of the epoxide to vitamin K. The thioredoxin-related redox-center in protein disulfide isomerase in the reduced state has been suggested to provide reducing equivalents to VKOR (Wajih *et al.*, 2007). VKOR is the target for coumarin derivatives such as warfarin, which is widely used as an anticoagulant. The reductase gene contains three exons and encodes a 163-amino acid protein. Missense mutations result in combined deficiency of VKD clotting factors type 2 due to inhibition of vitamin K recycling and subsequent inhibition of γ -carboxylation. Warfarin resistance also results from mutations in this gene.

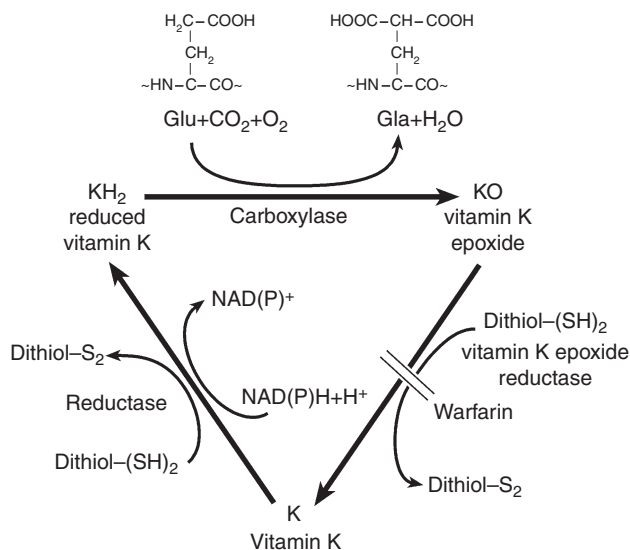


Figure 8.2 Vitamin K cycle.

The purified recombinant protein has been shown to be sufficient for the conversion of vitamin K epoxide to vitamin K (Chu *et al.*, 2006).

B. Mutations in epoxide reductase

Pauli *et al.* (1987) reported simultaneous occurrence of congenital deficiency of multiple VKD coagulation factors and chondrodysplasia punctata that they ascribed to deficiency in VKOR. The patient had features of warfarin embryopathy (Hall *et al.*, 1980). Combined deficiency due to defect in VKOR-multienzyme complex was also reported by Oldenburg *et al.* (2000).

V. MECHANISM OF γ -CARBOXYLATION

A. Amine rather than thiolate is the active base

During γ -carboxylation, a carbanion is generated at the γ -carbon. The pK_a for the deprotonation is estimated to be 25–28. Thus, a stronger base than that can be provided by amino acid functional groups is necessary to induce deprotonation. Dowd *et al.* (1995) proposed a base amplification hypothesis in which the oxygen adducts of reduced vitamin K would result in a strong base capable of deprotonating the γ -carbon. However, for O_2 addition, KH_2 has to be deprotonated (pK_a 9–10), and this can be achieved by functional groups of amino acid residues in the protein. From initial observations on the inactivation of the carboxylase by sulfhydryl modification with *N*-ethylmaleimide or *p*-hydroxy-mercuribenzoate (Canfield, 1987; Mack *et al.*, 1976; Suttie *et al.*, 1979) and the protection afforded by preincubation with KH_2 (Canfield, 1987; Pudota *et al.*, 2000), it was suggested that a thiolate anion may carry out the initial ionization of KH_2 . In contrast, studies on recombinant γ -carboxylase lacking cysteine residues and comparative sequence analysis of other γ -carboxylases by Rishavy *et al.* (2006) provide evidence suggesting that an amine (ϵ -NH₂ of lysine 218) was responsible for the initial ionization of KH_2 .

VI. PROPOSED TOPOLOGY OF γ -GLUTAMYL CARBOXYLASE

γ -Carboxylase is a microsomal integral membrane protein with the active site expected to be in the lumen (Bristol *et al.*, 1996; Carlisle and Suttie, 1980). Indeed, in a model proposed by Tie *et al.* (2000), active site residues K217 and K218 are present in the lumen. On the basis of the

determination of signal anchor sequences by *in vitro* translation/cotranslocation studies, Tie and coworkers proposed that the amino-terminus of the protein is on the cytosolic side of the endoplasmic reticulum membrane while the carboxy-terminus is in the lumen. This model has at least five transmembrane domains in contrast to seven putative transmembrane domains predicted by TOPPED II (von Heijne, 1992). In the absence of crystallographic or other direct observations, the results provide an important model for assessing the functional organization of the enzyme. Berkner (2005) pointed out the possibility that some of the putative transmembrane domains may be monotopic, thus serving as a binding site for hydrophobic substrates (e.g., vitamin K).

VII. SUBSTRATE RECOGNITION (PROPEPTIDE)

Precursors of VKD blood coagulation proteins contain a propeptide that is essential for efficient γ -carboxylation (Diguid *et al.*, 1986; Foster *et al.*, 1987; Jorgensen *et al.*, 1987). On the basis of the limited homology among propeptide sequences, Pan and Price suggested that propeptides may serve as the targeting sequence (Pan and Price, 1985). The ~ 18 -amino acids propeptide (γ -CRS) with conserved residues among the different γ -carboxylated proteins is cleaved in the mature protein (Kurachi and Davie, 1982). Phe is present at position -16 in all the propeptides except in Gas6 in which it is Leu and in Ci-Gla1 in which it is Ileu, Ala at -10 in all except bone Gla protein in which it is Gly, and hydrophobic residues at positions -17 , -15 , -7 , and -6 . Residues -18 , -17 , -16 , -15 , and -10 have been identified by site-directed mutagenesis as being the carboxylase recognition site of prothrombin (Huber *et al.*, 1990). Peptides, phenylalanine-leucine-glutamic acid-glutamic acid-valine (FLEEV) and phenylalanine-leucine-glutamic acid-glutamic acid-leucine (FLEEL), based on $+5$ to $+9$ residues of the Gla domains of prothrombin and factor VII are poor substrates with K_m in the range of 1 – 10 mM (Rich *et al.*, 1981; Suttie, 1993); however, with propeptides attached, the K_m is ~ 3 μ M (Ulrich *et al.*, 1988). Propeptide of factor X added in *-trans* at micromolar concentrations increased the level of carboxylation of a synthetic substrate boc-glu-glu-OMe, suggesting that the propeptide not only serves as a docking site but is also probably an allosteric effector (Knobloch and Suttie, 1987). Interaction of the propeptide with the enzyme contributes most to anchoring the substrate to the enzyme. Propeptides of different VKD proteins vary in their ability to enhance interaction with the enzyme (Stanley *et al.*, 1999). The relative affinities of propeptides were estimated from the K_i of the peptides in a factor IX-Gla domain carboxylation reaction. The K_i values of the propeptides of factor X, matrix Gla protein, factor VII, factor IX, PRGP I,

and protein S range from 2 to 35 nM, with factor X having the highest affinity; the affinities of prothrombin and protein C were ~ 100 -fold weaker than that of factor X. Changing residue -15 in protein C and -9 in prothrombin increases the affinity ~ 100 -fold. In bone Gla protein, the propeptide has poor affinity, 200,000-fold weaker than that of factor X, for the carboxylase (Stanley *et al.*, 1999) and probably uses an internal site to interact with the enzyme. This site on the enzyme also appears to be distinct from the propeptide binding site (Houben *et al.*, 1999). In matrix Gla protein, the γ -CRS is internal to the mature protein (Engelke *et al.*, 1991; Price *et al.*, 1987).

A. Propeptide binding site

Yamada *et al.* (1995) carried out photoinactivation studies of recombinant vitamin K-dependent carboxylase using [^{125}I]tyrosyl labeled benzoylphenylalanine-containing analogs of factor IX 19, a peptide containing -18 to $+1$ of factor IX. The label was attached to the amino terminal 259 residues of the γ -carboxylase molecule, suggesting propeptide binding to be located in that region. In contrast, based on the analysis of cross-linked product of factor IX 19 and factor IXQ/S (a recombinant peptide containing residues -18 to $+41$ of factor IX) to γ -carboxylase, Wu *et al.* (1997a) assigned the location of the propeptide binding site carboxy terminal to residue 438, probably between residues 438 and 507, located in the lumen of the endoplasmic reticulum. These two proposed regions though considerably distant in the linear sequence may be in close proximity in the 3-D sequence and result in the disparate observations depending on the cross-linking conditions.

VIII. STRUCTURE-FUNCTION RELATIONSHIP

Sugiura *et al.* (1996) carried out site-directed mutagenesis of clusters of charged residues in GGCX and concluded that K217 and/or K218 are either essential for catalytic activity or for maintaining the structure essential for catalytic activity; the regions around residues 234, 406, and 513 were important in propeptide binding, and the regions around residues 359 in catalysis. The propeptide binding region is in keeping with the observations of Wu *et al.* (1997a). It is particularly striking that either K217 or K218 has recently been suggested to provide the base responsible for the ionization of reduced vitamin K as the initial step in generating a strong base in the “base amplification” scheme (Rishavy *et al.*, 2006).

Mutations in clustered charged residues, K217A/K218A, R359A/H360A/K361A, and R513A/K515A, were demonstrated to impair enzymatic activity (Shimizu *et al.*, 1998). From results of mutations on individual

residues, the authors concluded that K-218, R-359, and H-360 were involved in the catalytic site while R-513 participated in propeptide binding.

Residues 343–355 of human γ -carboxylase bind VKD proteins. Mutations in Cys³⁴³ and Tyr³⁴⁵ lead to 85–90% loss of activity of the carboxylase (Pudota *et al.*, 2001). It has been suggested that these residues help to position Glu for carboxylation and help in processive carboxylation of substrates. *Drosophila* γ -glutamyl carboxylase (dGGCX), which lacks this region, efficiently carboxylates substrates containing factor IX propeptide (aa –18 to –1) covalently attached to the aa 1–10 of mature factor IX. The K_m for factor IX 28 (aa –18 to +10) is 8 μ M while that for factor IX 10 (aa +1 to +10) is 1.6 mM (Bandyopadhyay *et al.*, 2006). Clearly, this region is not necessary for carboxylation in dGGCX; however, its proposed role in processivity cannot be ruled out. It should be noted, however, that while conservation of residues among carboxylases from different sources suggest a functional role, the lack of conservation does not necessarily imply that they are not important.

Cys99 and Cys450 are essential for carboxylase function in huGGCX. While Tie *et al.* (2003) suggested a disulfide bridge between these residues and that Cys residues were not directly involved in catalysis (Tie *et al.*, 2004), Pudota *et al.* (2000) suggested that they were present as free thiols and that mutations resulted in perturbation of the active site (Rishavy *et al.*, 2004). The corresponding Cys residues are also essential for dGGCX and *Conus* γ -glutamyl carboxylase (cGGCX) activity (Bandyopadhyay *et al.*, 2006) (Stevenson, Bandyopadhyay, and Olivera, unpublished observations).

Naturally occurring mutations in γ -carboxylase have also provided additional information about the structure–function relationship. The first case reported by Brenner *et al.* (1990) was due to a missense mutation L394R that resulted in a defective glutamate-binding site (Mutucumarana *et al.*, 2000). Spronk *et al.* (2000) reported a case of a missense mutation in exon 11, W501S, resulting in a combined deficiency of VKD coagulation factors. The mutation appears to decrease the affinity of the enzyme for the propeptide of the substrate (Soute *et al.*, 2004). Rost *et al.* (2004b) identified mutations in exon 11 resulting in R485P and a mutation in the 3' splice site of intron 2 resulting in loss of exon 3. It was suggested that the R485P mutation affected either the propeptide or vitamin K binding sites in the γ -carboxylase enzyme. Rost *et al.* (2006) has also described an additional mutation H404P. Darghouth *et al.* (2006) reported three mutations, D31N, W157R, and T591K, in the γ -carboxylase gene. While the activity of the D31N mutation was similar to that of wild type, W157R and T591K resulted in activities that were 8% and 0% of wild type, respectively. W157 and its neighboring amino acid residues are conserved among carboxylases from different phyla. W157R has been suggested to be deficient in vitamin K catalysis. Thomas and Stirling (2003) identified a 14-base pair deletion in intron I (bases 1056–1069) of the γ -carboxylase gene and suggested that this may be a *cis*-acting region involved in γ -carboxylase expression.

IX. EXPRESSION OF GGCX DURING DEVELOPMENT

The expression of GGCX during development was investigated in rats. The carboxylase gene is 16.3 kb in length and contains 15 exons and 14 introns. Exon-intron splice junctions are conserved between the human and the rat enzymes (Romero *et al.*, 1998a). The expression of the gene is developmentally regulated (Romero *et al.*, 1998b). In rats, hepatocytes, where most of the VKD proteins are synthesized, express the enzyme late in gestation while central nervous system (CNS), mesenchymal, and skeletal tissues express the enzyme early in embryogenesis; this suggests that novel VKD proteins probably play an important role in central nervous system development. In this regard, it is important to note that CNS and skeletal abnormalities result from early fetal exposure to warfarin or to vitamin K deficiency. The mechanism of developmental regulation is not known.

In zebrafish, GGCX is expressed in the notochord, polster, YSL, and otic placode before being detected in the liver (Thisse *et al.*, 2001). In *C. intestinalis*, it is expressed at relatively constant levels in egg, larva, juvenile, and adults (Kulman *et al.*, 2006).

X. GLA-CONTAINING PROTEINS AND γ -CARBOXYLASE IN UROCHORDATE

Gla-domain proteins were identified (Jiang and Doolittle, 2003) and isolated (Kulman *et al.*, 2006) from the sea squirt *C. intestinalis* and tunicate *Halocynthia roretzi* (Wang *et al.*, 2003). The Gla domains are similar to that of the blood coagulation factors. However, none of the Gla domains are associated with the kringle domain, making it unlikely to be prothrombin precursors. Although associated with multiple EGF domains, they are not associated with protease domains. Four Gla domain-containing proteins have been characterized from *C. intestinalis* and a related ascidian. One of them, Ci-Gla1, is similar to Gla-receptor tyrosine kinase (Gla-RTK) from *H. roretzi* and contains a Gla domain typical of human VKD coagulation factors, a transmembrane domain, and a receptor tyrosine kinase domain similar to the ARK (adhesion-related kinase) family of receptor tyrosine kinases. Ci-Gla2 is similar to ligands for the Notch/Jagged family of receptors important in cell fate decision. Ci-Gla3 has a tandem EGF domain. Ci-Gla4 is similar to protein S and Gas6.

GGCX from *C. intestinalis* (ciG)GCX has been expressed by Kulman *et al.* (2006). It is 689 amino acids in length with carboxylation properties similar to those reported for the other carboxylases. Pairwise amino acid sequence comparison of ciGGCX with huGGCX reveals considerable divergence (48% identity). This is similar to the divergence between

huGGCX and cGGCX (49% identity). The presence of Gla domain in animals that predate fossil record of vertebrates and also lack the coagulation cascade suggests that γ -carboxylation and the associated coagulation factor Gla domain evolved to serve an essential function that was later recruited to the hemostatic process.

XI. *DROSOPHILA* γ -GLUTAMYL CARBOXYLASE

GGCX from *Drosophila* has been characterized (Li *et al.*, 2000; Walker *et al.*, 2001). However, carboxylated proteins have not been identified. Inactivation of the dGGCX by P-element insertion or simultaneous introduction of nonsense mutations at the amino- and carboxy-terminus by homologous recombination does not appear to cause any adverse effect on fly growth and reproduction under laboratory conditions (Bandyopadhyay *et al.*, 2006; Rong *et al.*, 2002). However, the gene function may be essential for coping with the environment in the wild.

dGGCX is 672 amino acids long and is foreshortened by 86 residues compared to the bovine enzyme. Its gene has two introns, with intron 2 being homologous to human intron 7. The carboxylase mRNA is present throughout embryogenesis with increased expression in 12–24-h embryos (Walker *et al.*, 2001). In adults, the highest concentration is present in the head (Li *et al.*, 2000). It is interesting to note in this connection that in rat CNS, RNA encoding the carboxylase is detected early in embryogenesis compared to its expression in hepatocytes (Romero *et al.*, 1998b). Mammalian propeptide-containing substrates are efficiently utilized by the *Drosophila* enzyme and probably interact with homologous sites on the enzyme (Bandyopadhyay *et al.*, 2006). This is also reflected in the poor carboxylation of substrates carrying a mutation of a hydrophobic residue of factor IX propeptide (F-16A) as observed with the mammalian enzyme. However, unlike the mammalian enzyme the enzymatic activity is not affected by propeptides presented in *trans*. The pair of Cys residues (Cys 99 and Cys 450, huGGCX coordinates) that are conserved in all carboxylases are essential for dGGCX activity. Substitution by alanine leads to inactivation of the enzyme. On the basis of genomic sequence analysis, Robertson (2004) identified the presence of VKOR in *Drosophila*. Mutation studies of the gene should yield additional insight into the role of vitamin K in *Drosophila* development.

XII. γ -CARBOXYLATED PEPTIDES IN *CONUS*

The venom of cone snails contains a multitude of small peptides, 12–30 amino acids in length (Olivera, 2002), a majority of which are ligands for ion channels (Terlau and Olivera, 2004). The peptides in general contain

multiple disulfide bonds and posttranslational modifications, including γ -carboxylation (Buczek *et al.*, 2005). These peptides contain γ -carboxyglutamate residues in the context of different sequences quite unlike that observed in the case of the mammalian VKD proteins (Table 8.1). Gla domains of proteins from tunicates and sea squirt to humans can be considered to have a single “signature” sequence while the γ -carboxylated *Conus* peptides provide examples of the multitude of possible Gla domains. The only families of *Conus* peptides that appear to have a “signature” Gla domain are the conantokins in that they have a conserved amino acid motif. It is interesting to note that like the Gla domain in vertebrate proteins, the Gla domain of conantokins and their propeptide sequences are part of the same exon (Walker, Garrett, Olivera, unpublished observations) and are cleaved off in the mature peptide. In contrast, in the other γ -carboxylated peptides, the propeptide and Gla domains are expected to be in separate exons (Olivera, 1997). When the propeptide and potential Gla domains are in separate exons, they can potentially evolve at different rates [as has been suggested for the evolution of other conotoxin families (Olivera *et al.*, 1999)], resulting in diversity of both Gla domains and carboxylation signals.

The role of the Gla residues in the structure–function relationship of conantokins has been extensively investigated. The $(i, i + 4)$ spacing of the Gla residues allows one Ca^{++} ion to coordinate to multiple Gla residues to form a stable α -helical structure (Blandl *et al.*, 2000; Klein *et al.*, 2001; Prorok and Castellino, 2001; Rigby *et al.*, 1997; Skjærbæk *et al.*, 1997). While the Ca^{++} -free conantokin-T (con-T) has considerable α -helical structure, Ca^{++} binding induces α -helicity in conantokin-G (con-G). Gla^3 and Gla^4 are essential for the interaction of con-G with *N*-methyl *D*-aspartate (NMDA) receptors (Warder *et al.*, 1998; Zhou *et al.*, 1996). Gla residues have also been suggested to stabilize structures favoring the synthesis of correct disulfide connectivity in the presence of Ca^{++} (Buczek *et al.*, 2005).

Table 8.1 shows a partial list of mature γ -carboxylated *Conus* peptides, Gla domains of human coagulation factors and other Gla-containing proteins, and Gla domains characterized in urochordates. The list is provided not to catalogue Gla domains but rather to emphasize the versatility of the enzyme. The non*Conus* Gla-proteins are relatively large; for example, PRGP2 has 153 amino acid residues; factor IX, 415 residues; Protein S, 635 residues; and Ci-Gla2, 1203 residues. The Gla domain consists of approximately the amino terminal 47 amino acids with 9–12 Gla residues (Stenflo and Dahlbäck, 2001). *Conus* peptides, in contrast, are between 12 and 50 amino acids. An exception is a 265-amino acid protein, GlaCrisp, containing a single Gla residue that has recently been isolated from the venom of *Conus marmoreus* (Hansson *et al.*, 2006).

The precursors of the *Conus* γ -carboxylated peptides also contain propeptide sequences that serve as γ -CRS for *Conus* γ -carboxylase (cGCX) isolated from the molluscivore, *C. textile* (Bandyopadhyay *et al.*, 1998;

Table 8.1 γ -Carboxylated Proteins

A. γ-Carboxyglutamate-containing peptides from <i>Conus</i>		
Superfamily-O		
Gla(1)-TxVI	MWG γ CKDGLTTCLOPS- γ CCS γ DC γ GS-CTMU	(Czerwiec <i>et al.</i> , 2006)
Gla(3)-TxVI	LCODYT γ OCSHAH- γ CCSUNCYNGHCTG	(Czerwiec <i>et al.</i> , 2006)
de7a	ACKOKNNLCAIT γ MA γ CCSGFCLYRCS#	(Aguilar <i>et al.</i> , 2005)
TxVIIA	CGGYSTYC- γ VDS- γ CCSDN CVRSYCTLF#	(Conticello <i>et al.</i> , 2001)
Tx-Rapid runner	CKTYSKYC- γ ADS- γ CCT γ QCVRSYCTLF#	(Cruz <i>et al.</i> , 1995)
γ PnVIIA	DCTSWFGRC-TVNS- γ CCSNSCDQTYC γ LYAFOS	(Fainzilber <i>et al.</i> , 1998)
Gla(2)-TxVIA	SCSDDWQYC γ SOTD-CCSWDCDVV-CS#	(Czerwiec <i>et al.</i> , 2006)
Bromosleeper	UATID γ C γ γ TCNVTFKTCGGOGDWQCV γ ACPV	(Craig <i>et al.</i> , 1997)
Lightsleeper	UFGH γ γ CTYULGPC γ VDDTCCSASC γ SKFCGLU	(Jimenez <i>et al.</i> , 2004)
Gla-TxX*	SCDS γ FSS γ FC γ RP γ γ S-CSCSTHTCCHWARRDQ-CMKPQRCISAQKGN#	(Brown <i>et al.</i> , 2005)
GlaMrII	SCDS γ FSS γ FC γ QP γ γ RICSCSTHVCCHLSSSKRDQCMTWNRCLSAQTGN	(Hansson <i>et al.</i> , 2004)
Superfamily-I		
Gla-TxXI*	CIP γ GSSCSSSGSCCHKSCCRWTCNQPClip#	(Brown <i>et al.</i> , 2005)
Superfamily-T		
GlaMrIII	FCCRTQ γ VCC γ AIKN#	(Hansson <i>et al.</i> , 2004)
GlaMrIV	CCITF γ SCC γ FDL	(Hansson <i>et al.</i> , 2004)
ϵ TxIX(tx5a)	γ CC γ DGUCCT $\dagger$ AAO	(Rigby <i>et al.</i> , 1999; Walker <i>et al.</i> , 1999)
Superfamily-A		
vcla	GCCSDORCNVDHP γ IC#	(Jakubowski <i>et al.</i> , 2004)
GID	IRD γ CCSNPACRVNNOHVC	(Nicke <i>et al.</i> , 2003)
κ A-MIVA	AO γ LVVTATTNCCGYNOMTICOOCMCTYSCOOKRKO#	(Santos <i>et al.</i> , 2004)
Superfamily-P		
Tx9a	GCNNSCQ γ HSDC γ SHCICTFRGCGAVN#	(Lirazan <i>et al.</i> , 2000)

(continued)

Table 8.1 (continued)

Conantokin		
Conantokin-G (Con-G)	GE $\gamma\gamma$ LQ γ NQ γ LIR γ KSN#	(Bandyopadhyay <i>et al.</i> , 1998)
Conantokin-T (Con-T)	GE $\gamma\gamma$ YQKML γ NLR γ AEVKKNA#	(Haack <i>et al.</i> , 1990)
Conantokin-R (Con-R)	GE $\gamma\gamma$ VAKMAA γ LAR γ NIAGCKVNCYP	(White <i>et al.</i> , 2000)
G-Scratcher	KFLSGGFK γ IVCHRYCAKGIAKEFCNCPD#	(Olivera <i>et al.</i> , 1992)
B. γ-Carboxyglutamate-containing proteins from various other animal species		
Snake factor Xa-like		
From <i>N. scutatus</i> <i>scutatus</i> and <i>T. carinatus</i>	SNSLF $\gamma\gamma$ IRPGNI γ R γ CI $\gamma\gamma$ KCSK $\gamma\gamma$ AR γ VF γ DN γ KTXXF	(Brown <i>et al.</i> , 2000)
Gla domain of human coagulation factors (Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)		
Prothrombin	ANT-FL $\gamma\gamma$ VRKGNL γ R γ CV $\gamma\gamma$ TCSY $\gamma\gamma$ AF γ AL γ S-STATDVFWAKYT	
Factor VII	ANA-FL $\gamma\gamma$ LRPGSL γ R γ CK $\gamma\gamma$ QCSF $\gamma\gamma$ AR γ IFKD-A γ R TKLFWISYS	
Factor IX	YNSGKL $\gamma\gamma$ FVQGNL γ R γ CM $\gamma\gamma$ KCSF $\gamma\gamma$ AR γ VF γ N-T γ RTT γ FWKQYV	
Factor X	ANS-FL $\gamma\gamma$ MKKGHL γ R γ CM $\gamma\gamma$ TCSY $\gamma\gamma$ AR γ VF γ D-SDKTN γ FWNKYK	
Protein C	ANS-FL $\gamma\gamma$ LRHSSL γ R γ CI $\gamma\gamma$ ICDF $\gamma\gamma$ AK γ IFQN-VDDTLAFWSKHV	
Protein S	ANS-LL $\gamma\gamma$ TKQGNL γ R γ CI $\gamma\gamma$ LCNK $\gamma\gamma$ AR γ VF γ NDP γ -TDYFYPKYL	
Protein Z	AGSYLL $\gamma\gamma$ LF γ GNL γ K γ CY $\gamma\gamma$ ICVY $\gamma\gamma$ AR $\gamma\gamma$ F γ N- γ VVTD γ FWRRYK	
Other Gla domain-containing proteins (Kulman <i>et al.</i> , 1997; Kulman <i>et al.</i> , 2001; Manfioletti <i>et al.</i> , 1993)		
BGP	YL-YQWLGAPVPYPDPL γ PRR γ VCLNPDCDELADHIGFQEAYRRF	(Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)

huPRGP1	ANGF- FγγIRQGNIγRγCKγγFCTFγγARγAFγNNγKTKγFWST	(Kulman <i>et al.</i> , 1997)
huPRGP2	ANHWDLγLLTPGNLγRγCLγγRCSWγγARγYFγDNTLTERFWES	(Kulman <i>et al.</i> , 1997)
TMG3	AN—EFLγγLRQGTIγRγCMγγICSYγVKγVFγNKγKTMγFW	(Kulman <i>et al.</i> , 2001)
TMG4	LLYNRFDLγLFTPGNLγRγCNγγLYNYγγARγIFVDγDKTIAFW	(Kulman <i>et al.</i> , 2001)
GAS6	FQVFγγAKQGHLYRγCVγγLCSRγγARγVFγNDPγTDYFY	(Manfioletti <i>et al.</i> , 1993)
Gla domain from <i>Ciona intestinalis</i> (Kulman <i>et al.</i>, 2006)		
Ci- Gla1	KNSWHHFγγAQQGNIγRγCIγγVCSWγγARγAFγTDVAGLNQFWNQYT	
Ci-Gla2	ANSWLRγγFQPGNIγRγCγγγRCSRγγARγAFγDDALTTγ-YWTRYT	
Ci-Gla3	ANSGF-γγFAKGNFγQγCVγγICNYγγLRγVYγNTAQTN-AKWDELT	
Ci-Gla4	SNRGFVLγγFSPGNQγRγCVγγICNYγγAFγISDNRIAN-KFWKKYT	
Gla domain from <i>Halocynthia roretzi</i> (Wang <i>et al.</i>, 2003)		
Gla-RTK	ANTASH- FγγIQQGNLYRγCYγγLCSFγγARγVFγTNIQDLNγ-FWAKYT	
C. Propeptides of Gla-containing proteins		
Gla(1)-TxVI	HSKENINFLLRKRAAD-R	(Czerwiec <i>et al.</i> , 2006)
Gla(2)-TxVIB	KIDFLSKGKADAQKQRKR	(Czerwiec <i>et al.</i> , 2006)
Gla(3)-TxVI	EKIKLLSKRKTDAEQQQR	(Czerwiec <i>et al.</i> , 2006)
Gla-TxX★	GRRRLIHMQK	(Brown <i>et al.</i> , 2005)
Gla-TxXI★	GKRAKLLEFFRQR	(Brown <i>et al.</i> , 2005)
tx5a	DDVPLSSLRDNLKRITRRLNIR	(Rigby <i>et al.</i> , 1999; Walker <i>et al.</i> , 1999)
tx9a	DNRRNLQSKWKPVSLYMSRR	(Lirazan <i>et al.</i> , 2000)
con-G	GKDRLTQMKRILKQRGNKAR	(Bandyopadhyay <i>et al.</i> , 1998)
hu Prothrombin	HVFLAPQQARSLQVRVR	(Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)

(continued)

Table 8.1 (continued)

hu FIX	TVFLDHEN ANKILNRPKR	(Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)
hu Protein C	SVFSSSER AHQVLRIRKR	(Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)
BGP	AAFVSKQEGSEVVKR PRR	(Furie <i>et al.</i> , 2003; Presnell and Stafford, 2002; Stenflo and Dahlbäck, 2001)
Gas6	AALLPARE ATQFLRRQRR	(Manfioletti <i>et al.</i> , 1993)
PRGP1	RVFLTGEK ANSILKRYPR	(Kulman <i>et al.</i> , 1997)
Ci-Gla1	ILIEKSE- ANRFLTRKLL	(Kulman <i>et al.</i> , 2006)

* From toxin-containing post-peptide.

amide; O: hydroxyproline; U: bromotryptophan; T† = O-glycosylated threonine

Bush *et al.*, 1999; Lirazan *et al.*, 2000; Walker *et al.*, 1999). Con-G, from the fish hunting snail *Conus geographus*, a 17aa peptide with five Gla residues, is a ligand for a subtype of glutamate receptors, NMDA receptors. The K_m for the Glu-con-G substrate is around 3 mM; in contrast, it is 28 μ M when the propeptide sequences (–20 to –1) are covalently linked to the substrate. When FLEEL is used as the substrate, the K_m changes from 400 to 4 μ M in the presence of the propeptide (Bandyopadhyay *et al.*, 1998).

In the case of ϵ -TxIX, a peptide obtained from *C. textile*, the K_m is reduced from $\sim$ 600 to $\sim$ 4 μ M by the presence of its propeptide sequences (–12 to –1) (Bush *et al.*, 1999). When the carboxylation of FLEEL was investigated in the absence and presence of the propeptide residues –1 to –20 of tx5a (ϵ -TxIX), the K_m decreased from 140 to 0.59 μ M. A novel configuration of γ -CRS with respect to the Gla domain has been identified (Brown *et al.*, 2005) in conotoxins Gla-TxX and Gla-TxXI isolated from the venom of *C. textile*. In the conventional arrangements seen in the case of the mammalian Gla proteins, the γ -CRS precedes the Gla domain, while in these isolates the Gla domain precedes the γ -CRS and establishes an instance of posttranslational modification at least in the case of these peptides, in contrast to a cotranslational modification plausible with the conventional arrangement. The γ -CRSs in Gla-TxX and Gla-TxXI designated “post-peptide” (Brown *et al.*, 2005) are also functional when positioned amino terminal to the target Gla residues. The post-peptide is also functional with heterologous substrates FLEEL and con-G. While Gla-TxXI-post-peptide is a good substrate (K_m 8 μ M) for cGCX prepared from venom duct microsomes, it is a poor substrate for bovine γ -carboxylase. The propeptides of the γ -carboxylated *Conus* peptides do not exhibit any obvious conservation among themselves or to the mammalian γ -CRSs.

Gla-containing peptides other than conantokins isolated from *Conus* contain multiple posttranslational modifications. They include disulfide bond formation, hydroxylation of proline, bromination of tryptophan, and isomerization of amino acids. An important question in regard to posttranslational γ -carboxylation in *Conus* is the order in which the multiple modifications are carried out and the associated signals. Since there are a large number of propeptides with no apparent sequence conservation, are there multiple propeptide interaction sites on the *Conus* γ -carboxylase, or adaptor molecules, or does the enzyme recognize a structural feature common to all the propeptide sequences? Czerwiec *et al.* (2006) pointed out possible important structural features, for example, the presence of conserved basic residues in the propeptide sequences of Gla-containing *Conus* peptides (Table 8.1C), α -helicity of the propeptide, and conserved residues with respect to the α -helix. Structure-function studies suggest that hydrophobic amino acids define γ -CRS of ϵ -TxIX (Bush *et al.*, 1999) and that basic amino acids may be less important in the function of γ -CRS of con-G (Bandyopadhyay *et al.*, 1998). In the absence of a conserved

γ -carboxylation signal sequence, it will be important to obtain 3-D structure of propeptide-Gla domain to define more stringent criteria for γ -carboxylation substrates.

XIII. *Conus* γ -GLUTAMYL CARBOXYLASE

cDNA encoding the *Conus* enzyme was isolated and expressed in insect cells in culture (Bandyopadhyay *et al.*, 2002; Czerwicz *et al.*, 2002). The enzyme is 811 amino acids long and its general properties, for example, vitamin K requirement, requirement for γ -CRS for efficient carboxylation, and epoxide activity, are similar to other VKD carboxylases. The gene organization of cGGCX is similar to that of the mammalian carboxylases. Of the 11 introns in *Conus* that we have mapped so far, the 10 intron-exon junctions in the mammalian gene are precisely conserved (Bandyopadhyay and Olivera, unpublished observations). The length and sequence of the introns have diverged depending on the evolutionary history of the animals; however, their conservation suggests that they are ancient, older than the Cambrian explosion (~540 million years ago) when mollusks and chordates first appear in the fossil record. Phylogenetic trees constructed using γ -carboxylase and a nuclear gene, elongation factor 1- α , are consistent, suggesting that the carboxylase is an ancient gene rather than being recently spread by horizontal transfer.

Carboxylated peptides isolated from *Conus* have 1–5 Gla residues. Whether the carboxylation is processive has not been determined. Compared to PT10 (amino acids 1–10 of mature human prothrombin) and factor IX 10 (amino acids 1–10 of mature human factor IX), proPT28 (amino acids –18 to +10 of human prothrombin) and profactor IX 28 (amino acids –18 to +10 of human factor IX), substrates containing the γ -CRS of mammalian VKD proteins, are efficiently carboxylated by the cGGCX, suggesting similar propeptide binding sites in the two enzymes. This is reflected in the conserved amino acids between residues 495 and –513 (huGGCX coordinates), a region postulated by Lin *et al.* (2002) to participate in propeptide binding.

	495	513
huGGCX	PFQRTSWVQPLLMDLSP	WR
dGGCX	PFE S TSWSL	PLLNELNHMR
cGGCX	VFK S TPWMMPLLVDLSN	WR

Amino acid alignment in proposed propeptide binding site among human, *Drosophila*, and *Conus* γ -carboxylases.

Additional experiments including double mutant cycle analysis involving the propeptide binding site sequences and propeptide sequences will be useful in identifying the partners in this interaction.

The versatility of the enzyme is obvious from the list of substrates carboxylated (Table 8.1); however, the specificity is reflected in the carboxylation pattern of the conantokins. Glu² is not carboxylated in any of the conantokins. Glu¹⁶ is also not carboxylated in con-T. Glu² is also not carboxylated in *in vitro* carboxylation of (–20 to +17)–con-G and this specificity is maintained in the absence of the propeptide (Fig. 8.3).

The pair of Cys residues (Cys 99 and Cys 450, huGGCX coordinates) that are conserved in all carboxylases are also essential for cGGCX activity. Their substitution by alanine results in inactivation of the enzyme (Stevenson, Bandyopadhyay, and Olivera, unpublished observations) (Fig. 8.4). Detailed structure–function studies of the cGGCX have not been reported. However, the availability of large number of natural substrates provides a powerful tool or unraveling substrate–carboxylase interactions.

XIV. FUTURE PROSPECTS

VKD γ -carboxylation is a posttranslational modification that occurs across different phyla, with the gastropods belonging to the genus *Conus* providing examples of the variety of amino acid sequence and structural

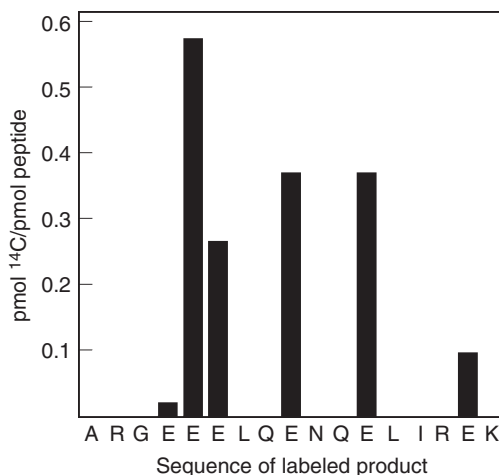


Figure 8.3 Specificity of γ -carboxylation. (–20 to +17)Glu–con-G. (GKDRLTQMK RILKQRGNKARGEEELQENQELIREKSN-NH₂) was carboxylated in presence of H¹⁴CO₃ using solubilized microsomes from *Conus textile* venom ducts. The product was isolated and digested with protease lysC. The labeled product was isolated and sequenced by automated Edman degradation. In a parallel analysis, [¹⁴C] was determined for each of the fractions. The x-axis shows the expected labeled product (ARGEEELQENQELIREK) and the y-axis, pmole of ¹⁴C incorporated at each position/pmole of peptide.

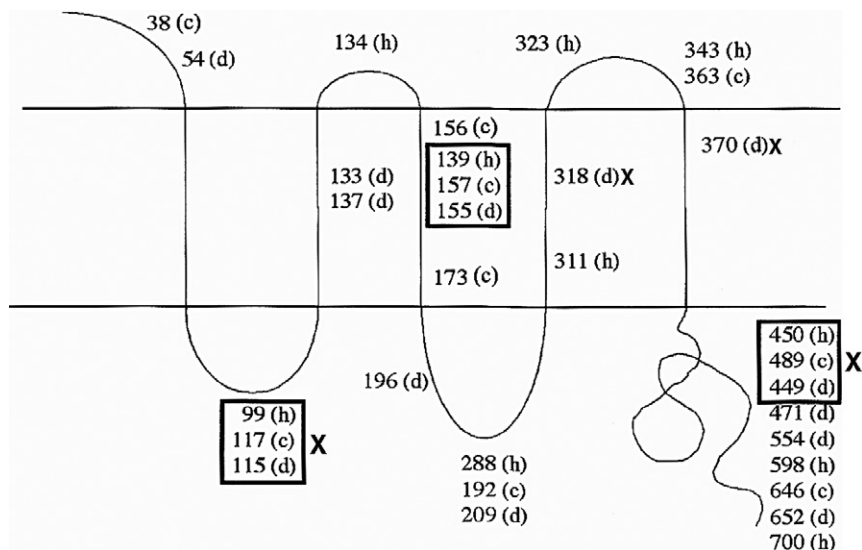


Figure 8.4 Proposed topologies of human, *Drosophila*, and *Conus* γ -glutamyl carboxylases after Tie *et al.* (2000). Positions of cysteine residues are shown for the human (h), *Conus* (c), and *Drosophila* (d) enzymes. Conserved residues are shown in a box. Mutation of residues at positions marked by X to alanine lead to reduction of activity of the enzyme (<10%).

contexts in which the modification is carried out. In analogy to *Conus*, short Gla-containing peptides may have been rather common in the early animals; their ability to interact with phospholipid membranes via Ca^{++} make them attractive candidates as signaling molecules for communication between cells. In addition, the bidentate Gla residues together with Ca^{++} may have participated in the reversible formation and disassembly of molecular scaffolds for intracellular or extracellular matrices. Specific sensitive reagents are necessary for the detection of Gla-containing peptides and proteins. Monoclonal antibodies to γ -carboxyglutamyl residues are being used with considerable success and promises to be a key reagent for this purpose (Brown *et al.*, 2000; Hansson *et al.*, 2006; Kulman *et al.*, 2006). However, the rules by which a peptide/protein is slated for modification is far from clear. Amino acid sequence of the γ -CRS as a criterion is equivocal. Structure-function studies of the large available repertoire of γ -carboxylated peptides together with their 3-D structures will provide the guidelines for predicting potential targets (Czerwicz *et al.*, 2006). With the ability to predict targets for modification, it will be possible to identify potential candidates from the ever-increasing information of genomic sequences to gain insight into the possible roles of this modification during early evolution.

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VITAMIN K-DEPENDENT ACTIONS OF GAS6

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Abstract

Gas6 (growth arrest-specific gene 6) is the last addition to the family of plasma vitamin K-dependent proteins. Gas6 was cloned and characterized in 1993 and found to be similar to the plasma anticoagulant protein S. Soon after it was recognized as a growth factor-like molecule, as it interacted with receptor tyrosine kinases (RTKs) of the TAM family; Tyro3, Axl, and MerTK. Since then, the role of Gas6, protein S, and the TAM receptors has been found to be important in inflammation, hemostasis, and cancer, making this system an interesting target in biomedicine. Gas6 employs a unique mechanism of action, interacting through its vitamin K-dependent Gla module with phosphatidylserine-containing membranes and through its carboxy-terminal LG domains with the TAM membrane receptors. The fact that these proteins are affected by anti-vitamin K therapy is discussed in detail. © 2008 Elsevier Inc.

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I. INTRODUCTION

The family of plasma vitamin K-dependent proteins was well established after the discovery of γ -carboxylation in 1974 (Stenflo, 2006). Plasma vitamin K-dependent proteins included the coagulation factors VII, IX, X, and prothrombin; and the natural anticoagulants protein C, protein S, and protein Z, all of them characterized by a preserved N-terminal Gla domain, which contained the γ -carboxyglutamic residues. Therefore, it was a surprise when in a screening for genes which expression was upregulated under conditions of growth arrest in embryonic mouse NIH 3T3 fibroblasts, the group of Dr. Schneider found a gene with a sequence related to the anticoagulant protein S (Manfioletti *et al.*, 1993; Schneider *et al.*, 1988). The gene was named growth arrest-specific gene 6 (*Gas6*; MIM#14456) and its expression was found to be increased around 30 times when the cell entered the G0 phase of the cell cycle. Shortly afterwards, the same group and others cloned the cDNA derived from the ortholog gene of human *PROS1* (MIM#176880) in the mouse (*Pros1*; MIM#19128) and the ortholog of *Gas6* in humans (*GAS6*; MIM#600441), demonstrating that these were two distinct entities, despite more than a 40% identity among protein S and *Gas6* protein sequences. Therefore, the short list of proteins dependent on vitamin K in plasma had a new genuine member.

II. GAS6 STRUCTURE

Gas6 is a multimodular protein containing several posttranscriptional modifications (Fig. 9.1). The main subject of this review is the γ -carboxylation of the N-terminal domain, the Gla module (Hansson and Stenflo, 2005). Gla modules of plasma γ -carboxylated factors have a common fold, which is preserved in *Gas6*. Together with protein S and prothrombin, *Gas6* contains a disulfide-bridged thumb loop, although it does not seem to be cleaved by the action of serine proteases, as it is the case of protein S, which is inactivated by thrombin- or factor Xa-specific cleavages in this region. From this connecting sequence, four epidermal growth factor-like modules are arranged in tandem, two of them with calcium-binding consensus sequences. The C-terminal region contains two domains similar to the globular folds of the laminin A chain (LG domains), a structure that is also found in the sex-hormone binding globulin (Sasaki *et al.*, 2002; Villoutreix *et al.*, 1997). The sequence similarity between protein S and *Gas6* is probably reflected in a close general structure, as suggested by modeling experiments using the available structures of *Gas6* or protein S fragments (Dahlbäck and Villoutreix, 2005).

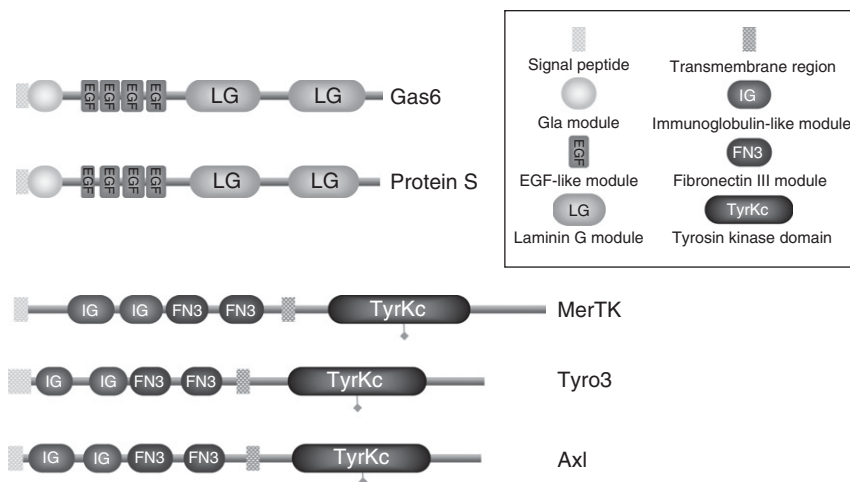


Figure 9.1 Schematic representation of Gas6, protein S, and the three members of the TAM receptor family, Tyro3, Axl, and MerTK. The domains were predicted with the Simple Modular Architecture Research Tool (SMART) program (<http://smart.embl.de/>) and represented using pfam graphical view of domain structure, from the Sanger Institute (<http://www.sanger.ac.uk/>). The tyrosine kinase active sites are indicated.

Despite this similitude, Gas6 and protein S seem to differ in function, as it is reflected by the tissue expression of *GAS6* and *PROS1*. Most plasma vitamin K-dependent factors have a gene expression restricted to liver, especially in the case of factor IX and factor X (Fig. 9.2). The expression of *PROS1* is not so specific for liver, and comparable levels of transcript can be found in kidney, lungs, or gonads. *GAS6* is unique among the genes of the family, because *GAS6* expression in liver is minor compared with other tissues, including heart, kidney, and lung (Fig. 9.2). The presence of Gas6 in plasma has been carefully evaluated. Although its presence can be detected, the concentration is quite small, around 20–50 ng/ml or 0.25 nM (Balogh *et al.*, 2005, 2006); or even lower according to other reports (Gibot *et al.*, 2007). In any case, the concentration of Gas6 is much lower than the rest of the vitamin K-dependent proteins of plasma, which range from 10 nM for factor VII to 1.5 μ M for prothrombin. The range of Gas6 concentration is also much lower than for protein S, which increases up to 400 nM during an acute-phase response (García de Frutos *et al.*, 1994).

III. CELLULAR EFFECTS OF GAS6

The function of Gas6 has been debated from its discovery. The fact that it was a cell arrest-regulated gene suggested that it could act as a factor involved in cellular homeostasis. Indeed, Gas6 was identified as a growth

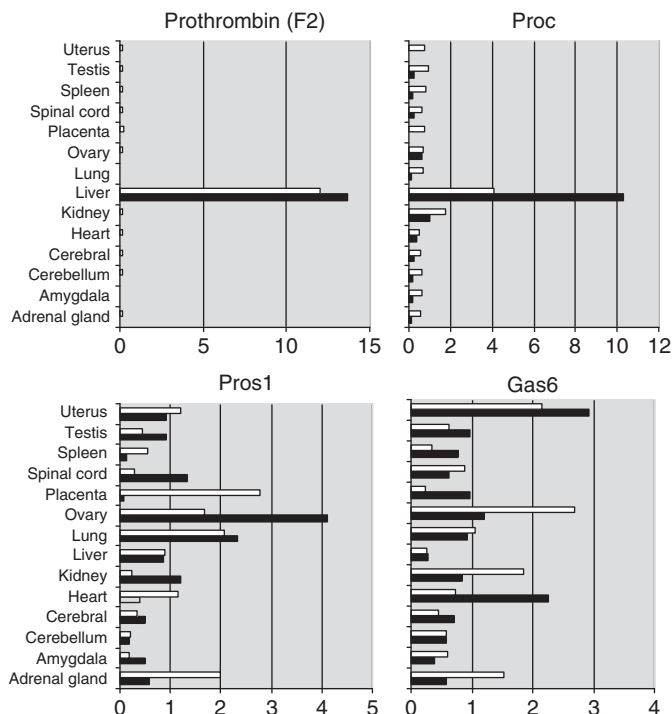


Figure 9.2 Expression of selected plasma vitamin K-dependent proteins in a subset of human (white bars) and mouse (black bars) organs. Data were obtained from the SymAtlas microarray expression database and represent the relative value of expression of each gene (<http://symatlas.gnf.org/Su> *et al.*, 2002).

factor and a rescue factor from apoptosis on different cell types, specifically in cultured cells deprived of serum (Goruppi *et al.*, 1996; Li *et al.*, 1996; Nakano *et al.*, 1995). Growth factors are major effectors of cell differentiation, duplication, and survival. Their effect is mediated by the interaction with specific cell membrane receptors, most of them with a tyrosine kinase activity (receptor tyrosine kinases or RTKs). RTKs are a large group of genes with a highly conserved intracellular tyrosine kinase domain. Intracellular signaling is triggered upon ligand binding, which induces dimerization/oligomerization of the receptor transducing the signal to the intracellular portion of the molecule and leading to activation of the tyrosine kinase activity. An essential early substrate of tyrosine phosphorylation is the RTK itself. RTKs are classified according to their extracellular domain, generally composed of a modular structure (Fig. 9.1). Members of a subgroup of RTKs often bind common or similar ligands.

A crucial discovery in the understanding of the biology of Gas6 was the discovery that it was a ligand of a previously orphan RTK, Axl. Axl was part

of a three-member family of receptors composed of Axl, Tyro3, and MerTK, which had been cloned by different groups and given different names: Axl (ARK, Tyro7, Ufo), Tyro3 (Sky, Rse, Brt, Dtk, Tif, Etk2, Rek), and MerTK (Mer, c-Eyk, Nyk, Tyro12). The architecture of these receptors is preserved, and it is constituted by two immunoglobulin domains followed by two fibronectin type III (Fig. 9.1). This RTK family is named TAM from the first letter of the three components (Tyro3, Axl, and MerTK).

From the early studies of Gas6 action on cultured cells, the antiapoptotic effect of Gas6 has been found in many experimental settings, as well as in different species. This has been observed in almost all cell types and lines tested, including NIH3T3 fibroblasts (Bellosta *et al.*, 1997; Goruppi *et al.*, 1996, 1999), vascular smooth muscle cells (VSMCs, Konishi *et al.*, 2004; Melaragno *et al.*, 2004; Nakano *et al.*, 1996), endothelial cells (Hasanbasic *et al.*, 2004; Healy *et al.*, 2001; O'Donnell *et al.*, 1999), chondrocytes (Loeser *et al.*, 1997), neurons (Allen *et al.*, 1999; Yagami *et al.*, 2003), oligodendrocytes (Shankar *et al.*, 2003), hepatocytic precursors (Couchie *et al.*, 2005), epithelial cells (Valverde *et al.*, 2004), and different types of cancer cells (Sawabu *et al.*, 2007; Vajkoczy *et al.*, 2006; van Ginkel *et al.*, 2004). The antiapoptotic effect was observed after treatment with different stimuli inducing cell death, including serum deprivation, but also more specific effectors as TNF- α (Bellosta *et al.*, 1997; O'Donnell *et al.*, 1999; Shankar *et al.*, 2006; Valverde *et al.*, 2004), changes in pH (D'Arcangelo *et al.*, 2002), oxidative stress (Konishi *et al.*, 2004; Valverde, 2005), soluble phospholipase (Yagami *et al.*, 2003), or the amyloid peptide (Yagami *et al.*, 2002). The antiapoptotic effect of Gas6 requires protein phosphorylation, as general inhibitors as staurosporine abolished it (Bellosta *et al.*, 1997), and in most cases, the PI3K pathway has been demonstrated to be crucial for this effect (see below). Interestingly, ultraviolet light exposure, which induces apoptosis in a p53-dependent manner, was not affected by Gas6/Axl (Bellosta *et al.*, 1997).

While the antiapoptotic effect of Gas6 has been repeatedly documented, it has not been so easy to study the mitogenic activity of the molecule. In certain cell types, a mitogenic effect has been described as a potentiating effect on other factors. Indeed, Gas6 was cloned as a growth potentiating factor for prothrombin on VSMCs (Nakano *et al.*, 1995). Gas6 stimulates also the mitogenic effect of EGF on cardiac fibroblasts (Stenhoff *et al.*, 2004), and has been also reported to stimulate heregulin mitogenic activity on Schwann cells (Li *et al.*, 1996). The mitogenic effect observed in these experiments did not seem to be dependent on PI3K activation, rather it was mediated through activation of the Ras/ERK pathway in most cases (Fridell *et al.*, 1996; Goruppi *et al.*, 1996, 1997, 1999; Sainaghi *et al.*, 2005). Nevertheless, significant differences have been reported depending on the cell type and context studied (Goruppi *et al.*, 2001), which could reflect

differences in the span of Gas6 receptors expressed by the cells studied. Indeed, Gas6 has been shown to induce mesangial cell proliferation in cultured cells in a STAT3-dependent manner that correlates with Axl activation (Doi, 2001; Yanagita *et al.*, 2001b). It is possible that in certain cell types or in undifferentiated cancer cells, Gas6/Axl would have a more clear mitogenic effect than in other cell types (Sainaghi *et al.*, 2005).

A further cellular action of Gas6 is the induction of chemotaxis of VSMCs (Fridell *et al.*, 1998). An effect on cell migration has been suggested to play a role on gonadotropin releasing hormone (GnRH) neurons migration into the hypothalamus during development (Allen *et al.*, 2002; Nielsen-Preiss *et al.*, 2007). In certain tumor types, migration and invasiveness has been correlated with the expression of Gas6 receptors, in particular Axl (Shieh *et al.*, 2005), and Gas6-dependent signaling through Axl has been shown to promote invasiveness of glioma cells in animal models (Vajkoczy *et al.*, 2006).

Another cellular effect which has been demonstrated for Gas6 is the targeting of apoptotic cells and induction of phagocytosis by macrophages and other phagocytic cell types (Ishimoto *et al.*, 2000). The induction of phagocytosis has been shown to be specific of apoptotic cell removal or phosphatidylserine containing liposomes, mimicking the situation in the cellular membranes of apoptotic cells (Hall *et al.*, 2001; Ishimoto *et al.*, 2000; Wu *et al.*, 2005, 2006), while Gas6 does not influence the phagocytic capacity of these cells on bacteria, yeast, or particulate substrates (Scott *et al.*, 2001; Wu *et al.*, 2006). The mechanism of Gas6 action could be linked to an integrin cooperative recognition of apoptotic cells and subsequent activation of macrophages (Wu *et al.*, 2005). Unraveling the mechanisms and molecules implicated in the phagocytosis of apoptotic cells and the resolution of inflammation has demonstrated that it is a specific process (Wu *et al.*, 2006). The new term efferocytosis has been proposed to designate the specific removal of apoptotic cells by phagocytic cells (Vandivier *et al.*, 2006). Related to the effect on apoptotic clearance, Gas6 has been implicated in the formation of cellular aggregates mediated by Axl. Initially, it was shown that the mouse Axl receptor was able to induce cellular aggregation in the absence of substrates when expressed as a transgene in *Drosophila* cells (Bellosta *et al.*, 1995). Afterwards, the formation of aggregates was found to be dependent on the presence of Gas6 in mammalian cells (McCloskey *et al.*, 1997). Nevertheless, recent studies have shown that the soluble form of Axl is able to induce signaling through membrane-bound Axl when coated on plates, indicating that a possible homophilic interaction among Axl molecules independent of ligands is potentially relevant (Budagian *et al.*, 2005a).

Some studies have found a regulatory role of Gas6 on cell activation and differentiation. Cells from innate immunity downregulated the expression of inflammatory cytokines after Gas6 stimulation, a function that has been

mainly attributed to the interaction with MerTK (Camenisch *et al.*, 1999; Correll *et al.*, 2004; Li *et al.*, 2006; Sharif *et al.*, 2006). In contrast, MerTK activation seems to mediate IL-8 induction in prostate cancer cells (Wu *et al.*, 2004), inducing its differentiation. Induction of differentiation by Gas6 has also been reported in the maturation process of natural killer cells (Caraux *et al.*, 2006; Walzer and Vivier, 2006) and adipocytes (Maquoi *et al.*, 2005; Shugart *et al.*, 1995). Gas6 has shown an activity as modulator of differentiation models on cells of the vessel wall, including the inhibition of osteogenic differentiation of pericytes (Collett *et al.*, 2003) and the differentiation of VSMCs into foam cells, as shown by the induction of the expression of specific markers as the scavenger receptor A (Ming *et al.*, 2001; Murao *et al.*, 1999). This property could have an important role in the cellular homeostasis of the vasculature after damage (Melaragno *et al.*, 1999).

IV. INTERACTION WITH TAM RECEPTOR MOLECULES AND SIGNAL TRANSDUCTION IN THE GAS6/TAM LIGAND/RECEPTOR SYSTEM

The first report proposing Gas6 as a ligand for TAM receptors showed that Gas6 engagement induced autophosphorylation of the Axl receptor (Varnum *et al.*, 1995). The active receptor recruits components of several signaling pathways. One of the first molecules to be reported as recruited after Gas6 stimulation of the Axl RTK were PI3K subunits (Goruppi *et al.*, 1997). Furthermore, docking sites in Axl intracellular kinase domain for PI3K subunits, Grb2, c-Src, and Lck were localized in phosphorylated tyrosines Y821, Y779, and Y866 (Braunger *et al.*, 1997). Other partners of Axl have been proposed using the yeast two-hybrid system, including SOCS-1, Nck2, and C1-TEN, proteins containing Src homology 2 domains (SH2), apart from confirming the previously found interactions with PI3K components and Grb2 (Hafizi *et al.*, 2002).

To date, almost all studies have found a link between PI3K activation and Gas6/Axl effect on cells. The activation of the PI3K pathway has been shown to be indispensable for the antiapoptotic effect of Gas6 on different cell types (Allen *et al.*, 1999; Goruppi *et al.*, 1999; Hasanbasic *et al.*, 2004; Lee *et al.*, 2002). The cell survival effect is blocked by pharmacological inhibitors of the PI3K pathway (Allen *et al.*, 1999; Goruppi *et al.*, 1997, 1999; Hasanbasic *et al.*, 2004; Melaragno *et al.*, 2004). Axl mutants which are not capable of interacting with PI3K subunits also block the survival effect of Gas6 on cells (Melaragno *et al.*, 2004; Vajkoczy *et al.*, 2006). Several isoforms of PI3K have been shown to interact with Axl, and they could potentially lead to discrete signaling pathways from the receptor (Braunger *et al.*, 1997; Hafizi *et al.*, 2002), and this could have different implications

depending on the cell type studied. The activation of PI3K initiates the activation of the antiapoptotic Akt/PKB pathway, while downstream inhibition of PI3K also blocks the antiapoptotic effect of Gas6. In this context, it is interesting that C1-TEN, a protein phosphatase similar to the PI3K/Akt/PKB inhibitor PTEN, was also found to interact with Axl, although the potential regulatory role of the Axl/C1-TEN interaction has not been elucidated (Hafizi *et al.*, 2002). Downstream substrates of Akt/PKB shown to be modulated by Gas6 include the antiapoptotic phosphorylation of Bad (Goruppi *et al.*, 1999; Lee *et al.*, 2002). Further, the PI3K/Akt/PKB pathway activates NF κ B transcriptional activity, increasing the concentration of Bcl-2, among other effects (Demarchi *et al.*, 2001; Hasanbasic *et al.*, 2004). Another effector of the Akt/PKB pathway in the context of Gas6 action could be glycogen synthase kinase-3 (GSK3), which has been shown to mediate contact inhibition by interacting with the Wnt pathway (Goruppi *et al.*, 2001).

Interaction of the Axl kinase domain with the adaptor protein Grb2 (Braunger *et al.*, 1997; Fridell *et al.*, 1996) seems to be the necessary link with Ras-mediated activation of the mitogen-activated protein kinase (MAPK) pathway of the extracellular regulated kinases (ERKs). In this context, it is interesting to note that earlier studies found an Axl-dependent phosphorylation of the ribosomal protein S6 kinase (RPS6KB1), the activity of which leads to an increase in protein synthesis and cell proliferation (Goruppi *et al.*, 1997). As mentioned before, the activation of the Ras/ERK pathway is essential for the observed mitogenic activity of Gas6 (Fridell *et al.*, 1996; Goruppi *et al.*, 1996; Stenhoff *et al.*, 2004). A related adapter protein containing SH2 binding domains, Nck2, was also found to interact with Axl in the two hybrid system (Hafizi *et al.*, 2002). This interaction is interesting, as it could provide a link of Axl activation with integrin-linked kinases, affecting in this way the cytoskeletal dynamics through focal adhesions and providing a cooperative pathway with integrin signaling (Hafizi and Dahlbäck, 2006), as has been shown for MerTK (Finnemann and Nandrot, 2006; Nandrot and Finnemann, 2006; Wu *et al.*, 2005). This process is probably mediated by interaction with Vav1 and small GTPases of the Rho family (Mahajan and Earp, 2003). The activation of another phosphatase, SHP-2, has been shown to be essential for the effect of Gas6/Axl on inhibition of vascular endothelial growth factor receptor 2 (VEGFR2, FLK1, KDR) signaling on endothelial cells (Gallicchio *et al.*, 2005).

The cross talk between Gas6/Axl and KDR illustrates the way in which cells integrate signals from different stimuli, thereby coordinating and refining complex responses. An interesting example of this cooperation has been provided by the study of IL-15 antiapoptotic effect. In this case, a direct interaction of the Axl receptor with the receptor for IL-15 has been demonstrated and shown to be relevant for the signaling cascades of both

receptors. IL-15 is able to transactivate Axl through its specific receptor IL-15R (Budagian *et al.*, 2005b). In an Axl-dependent manner, IL-15 is able to activate the Akt/PKB pathway and induce survival to TNF- α in Axl-transfected cells. Differences among the cells studied suggest that specific mechanisms related to putative partners of the Gas6/Axl system could be acting in different cell types leading to relevant changes in the response to Gas6. Furthermore, these studies demonstrate that differences in the extracellular part of the receptors could contribute not only to ligand recognition, but also to partner recruitment.

Other signaling pathways activated by Gas6/Axl have been studied. Some studies found a transient activation of other MAPKs, including JNK/SAPK and p38 (Bellosta *et al.*, 1997; Goruppi *et al.*, 1999). The latter activation has been shown to be important in the migration of GnRH neurons (Allen *et al.*, 2002; Nielsen-Preiss *et al.*, 2007). Finally, the Janus kinase (JAK) pathway is also affected by Gas6/Axl, leading to activation of signal transduction and activators of transcription factors (STATs). As mentioned earlier, the mitogenic effect of Gas6 on mesangial cells was shown to be mediated by activation of STAT3 through stimulation of Axl (Yanagita *et al.*, 2001b). This effect has been shown to be important in the development of certain models of renal disease (Yanagita, 2004; Yanagita *et al.*, 2002).

The signaling pathways initiated by Tyro3 have been comparatively less studied to those initiated by Axl or MerTK. Apart from autophosphorylation of the receptors, Tyro3 and MerTK have been found to bind and activate Src kinase (Toshima *et al.*, 1995; Wu *et al.*, 2005), similarly as was observed for Axl (Braunger *et al.*, 1997). The activation of regulatory subunits of PI3K has also been demonstrated for Tyro3 (Lan *et al.*, 2000). In osteoclasts, Gas6 has been shown to stimulate Tyro3 through a Ras/ERK-mediated mechanism (Katagiri *et al.*, 2001). Interestingly, in this cell type, no activation of alternative MAPK pathways was reported (Katagiri *et al.*, 2001). Constitutively active forms of MerTK have been shown to autophosphorylate residues Y749, Y753, and Y754 and, upon activation, phosphorylate PLC- γ , PI3K, Shc, Grb2, Raf-1, and ERK downstream the receptor (Ling *et al.*, 1996). In a second study, the binding to Grb2 was shown to be dependent on Y872, and it is essential in the PI3K activation leading to an antiapoptotic signal through activation of the NF κ B pathway (Georgescu *et al.*, 1999). In contrast to the mitogenic activity observed with Axl activation, MerTK kinase domain rescued cells from apoptosis, but did not induce proliferation, despite activating ERK, PI3K, and p38 pathways (Guttridge *et al.*, 2002). PLC- γ has been shown to interact and be a substrate of MerTK, and is essential in phagocytosis of apoptotic cells (Todt *et al.*, 2004). Again, PLC- γ has also been shown to bind Axl, where it could also be implicated in a pathway affecting the dynamics of the cytoskeleton (Braunger *et al.*, 1997; Nielsen-Preiss *et al.*, 2007).

As was shown for Axl, MerTK has been shown to activate the Jak/STAT pathway leading to increased activation of Stat3 and increased transformation efficiency (Besser *et al.*, 1999). In this context, it is interesting to note that another partner of Axl found by the yeast two-hybrid assay was SOCS-1, a negative regulator of cytokine signaling acting through the inhibition of Jak phosphorylation, and therefore inhibiting the Jak/STAT pathway (Hafizi and Dahlbäck, 2006). Although not studied, it is possible that a similar interaction is provided by MerTK. From these studies, the general view shows that due to the high conservation in the intracellular sequence of the TAM receptors, most interacting partners are common, although each receptor in the family show marked differences in the biological function of the three receptors.

V. ROLE OF THE GAS6/TAM SYSTEM IN VASCULAR BIOLOGY

One of the first discoveries linking Gas6 with the biology of vascular vessels was the already mentioned cloning of a growth potentiating factor of VSMCs, shown to be Gas6 (Nakano *et al.*, 1995). Axl was also cloned from the rat carotid artery after mechanical damage. Both Gas6 and Axl increased their expression in this model, mainly in the developing neointima up to 14 days after damage, the increase in Gas6 preceding that of the receptor (Melaragno *et al.*, 1998, 1999). Given the earlier studies showing an anti-apoptotic and mitogenic effect of the Gas6/Axl system in cultured cells, it was supposed that proliferating cells would need the signaling through Gas6/Axl in order to migrate and/or proliferation of VSMCs. The clear effect observed in VSMCs migration in cell culture (Fridell *et al.*, 1998) indicates that Gas6 could also play a role in these systems *in vivo*, affecting neointima migration as well as the organization of the atherosclerotic plaque (Lutgens *et al.*, 2000). Indeed, Axl-deficient mice show a decreased neointima formation after mechanical injury (Konishi *et al.*, 2004; Korshunov *et al.*, 2006). Nevertheless, the Axl knockouts showed not only a decreased number of proliferating muscular cells, and an increase in apoptosis, but also less macrophages and neutrophils (Korshunov *et al.*, 2006). A parallel situation has been reported in atherosclerosis of Gas6 $-/-$ animals, where a lower content of lymphocytes and macrophages has been found (Lutgens *et al.*, 2000).

Apart from the effect on VSMCs, a role of Gas6 on the endothelium has been suggested from its antiapoptotic effect in culture (D'Arcangelo *et al.*, 2002; Hasanbasic *et al.*, 2004; Healy *et al.*, 2001; O'Donnell *et al.*, 1999). On one hand, the decreased content of macrophages and neutrophils in Axl $-/-$ mice during intimal thickening suggests differences in cell recruitment, a process mediated by endothelial/leukocyte interactions (Korshunov *et al.*, 2006).

On the other hand, the addition of Gas6 has been shown to inhibit adhesion of granulocytes to endothelial cells (Avanzi *et al.*, 1998). These studies suggest an effect of the Gas6/TAM system on endothelial function beyond the antiapoptotic effect observed in cultured endothelial cells.

Recently, an important function of Gas6 has been described in relation to the differentiation of pericytes. Vascular pericytes undergo osteogenic differentiation *in vivo* which may be implicated in diseases involving ectopic calcification and osteogenesis, a process that was inhibited by Gas6/Axl signaling (Collett *et al.*, 2003). This effect is not completely surprising considering several reports implicating the Gas6/TAM system in osteoclast function (Katagiri *et al.*, 2001; Kawaguchi *et al.*, 2004; Nakamura *et al.*, 1998). It has recently been reported that the induction of calcification of cultured VSMCs is inhibited by Axl (Collett *et al.*, 2007), and the reversion of the process by statins was also mediated by activation of the Gas6/Axl pathway (Son *et al.*, 2006, 2007). In this context, the linkage between genetic variants in *GAS6* and stroke could be due to the effect of Gas6 on vascular calcification, a process clearly associated with the risk of stroke (Munoz *et al.*, 2004).

Platelets are key players in the cardiovascular system, not only providing primary hemostasis, but also mediating inflammatory responses and cellular interactions. Gas6 is found in platelets from rats and mice (Angelillo-Scherrer *et al.*, 2001; Ishimoto and Nakano, 2000), and animals deficient in Gas6 are protected in thrombosis models and their platelets are less prone to activation (Angelillo-Scherrer *et al.*, 2001). Both MerTK and Axl and to a lesser extent Tyro3, have been proposed as mediators of this function using knockout mice and antibody inhibition (Angelillo-Scherrer *et al.*, 2001, 2005; Chen *et al.*, 2004; Gould *et al.*, 2005). However, further studies showed that the concentration of Gas6 in human platelets was lower than the concentration found in plasma, and did not affect platelet response (Balogh *et al.*, 2005; Clauser *et al.*, 2006; Borgel *et al.*, 2006). Still, Gas6 could affect antiplatelet drug responsiveness (Burnier *et al.*, 2006). Recently, the extracellular part of MerTK has been shown to be able to inhibit platelet aggregation and pulmonary embolism, further emphasizing that, at least in mice, the Gas6/TAM pathway is involved in hemostasis (Sather *et al.*, 2007).

VI. GAS6 IN INNATE IMMUNITY: A MODULATOR OF THE INFLAMMATORY RESPONSE

Cells from the immune system express TAM receptors and their ligands. TAMs are detected on antigen-presenting cells from both peripheral blood or spleen, thymus, and lymph nodes either with specific antibodies after cell sorting or by *in situ* hybridization (Behrens *et al.*, 2003; Lu and Lemke, 2001; Mahajan and Earp, 2003; Neubauer *et al.*, 1994; Sen *et al.*, 2007). In contrast,

TAMs are not expressed in granulocytes or blood lymphocytes (Graham *et al.*, 1995; Neubauer *et al.*, 1994). It is likely that most monocytic cells express more than one TAM receptor, although it cannot be excluded that certain populations express only specific receptors (Lemke and Lu, 2003; Lu *et al.*, 1999). The function of TAM receptors in the immune system is dramatically illustrated in animals deficient in all three receptors (Lu *et al.*, 1999; Lu and Lemke, 2001). TAM-deficient animals survive and do not present defects during early life. Peripheral lymphoid organs are normal, indicating a broadly normal hematopoiesis, but from one month of age, spleens and lymph nodes grow disproportionately, reaching 10 times the weight of these organs in wild-type mice. While all three mutations are involved in this phenotype, the MerTK mutation produces the largest effect (Lu and Lemke, 2001; Scott *et al.*, 2001). Animals deficient in MerTK present defects in monocyte function, including an enhanced sensitivity to endotoxic shock (Camenisch *et al.*, 1999), a delayed clearance of apoptotic cells, and splenomegaly (Cohen *et al.*, 2002; Scott *et al.*, 2001). TAM- or MerTK-deficient animals show signs of autoimmunity, with features resembling certain human autoimmune pathologies including serum autoantibodies against DNA, collagen and antiphospholipid antibodies (i.e., anticardiolipin antibodies), and lymphocyte activation and hyperproliferation (Lu and Lemke, 2001; Lu *et al.*, 1999). The fact that lymphocytes become activated in TAM-deficient mice, despite not expressing the TAM receptors, indicates that autoimmunity in these animals is mediated through a cell-dependent mechanism. Probably, the lack of an inhibitory signal arising from Gas6/TAM lowers the activation state of antigen-presenting cells subsequent to an initial immune response. Multiple observations, made in both the MerTK single mutants and the triple TAM mutants, are consistent with this suggestion. First, macrophages from MerTK mutants and TAM-deficient animals, when challenged with bacterial lipopolysaccharide (LPS), express unusually high levels of activated NF κ B, and the mice are hypersensitive to LPS-induced endotoxic shock, tissue damage, and death as a result of the excessive production of TNF- α (Camenisch *et al.*, 1999; Cohen *et al.*, 2002; Lu *et al.*, 1999; Scott *et al.*, 2001; Sen *et al.*, 2007). Markers of antigen-presenting cell activation, including cell-surface expression of MHC class II and the B7 coreceptors, are constitutively activated in the TAM mice in comparison to wild-type mice and are consistently hyperactivated in response to LPS challenge (Lu and Lemke, 2001).

In addition to lymphocyte hyper-responsiveness, the second key feature displayed by TAM mutants that almost certainly contributes to their development of autoimmune disease is that macrophages fail to clear apoptotic cells. This phenomenon has been most thoroughly analyzed in the MerTK single mutants (Cohen *et al.*, 2002; Li *et al.*, 2006; Scott *et al.*, 2001; Sen *et al.*, 2007). The delayed clearance of apoptotic cells is dependent on the ability of the vitamin K-dependent protein S and Gas6 to bind to phosphatidylserine and acidic phospholipids through their amino-terminal Gla domains (Cohen *et al.*, 2002; Nakano *et al.*, 1997a; Scott *et al.*, 2001;

Sen *et al.*, 2007; Wu *et al.*, 2006). Probably, the lack of efferocytosis is enhanced in the TAM-deficient animals by an increased cell death due to the lack of an antiapoptotic rescue signal, as has been shown for Gas6 in cell cultures. *In vivo*, an increased apoptotic cell death has been observed in retina, brain, gonads, and other organs, suggesting that TAMs play a role in maintaining the cellular homeostasis of organs beyond their function in the immune response (Lemke and Lu, 2003; Lu and Lemke, 2001; Lu *et al.*, 1999).

VII. FURTHER FUNCTIONS OF GAS6/TAM RECEPTORS IN CELLULAR HOMEOSTASIS

The role of Gas6 on cellular homeostasis, related to its mitogenic and antiapoptotic properties, has been implicated in several organs and models of disease. Considering the tissue distribution of both ligands and receptors, it was expected that the Gas6-Protein S/TAM system would have implications in neural tissues. Tyro3 single mutants exhibit compromised central nervous system (CNS) function [diminished hippocampal long-term potentiation (LTP)] as young adults and, when aged, they exhibit neural degeneration accompanied by seizures and hind limb paralysis (Lu *et al.*, 1999). This phenotype is clearly more severe in the animals deficient in all TAM receptors (Lu *et al.*, 1999). Gas6 has also been implicated in the cellular homeostasis of the kidney after certain damages, including nephrotoxic nephritis and diabetic glomerulopathies (Nagai *et al.*, 2005; Yanagita *et al.*, 1999, 2001a, 2002). The mechanism of action seems to be mediated by the mitogenic effect of Gas6 on mesangial cells. Mesangial cell proliferation is a general characteristic of a variety of glomerular diseases (Yanagita *et al.*, 1999; 2001a, b). A third system where Gas6 function has been implicated is in the development of germinal cells (Matsubara *et al.*, 1996). Most TAM-deficient animals are infertile due to the degeneration of all germ cells in the gonads (Lu *et al.*, 1999). These observations support the view that Gas6 could act as a factor maintaining at least certain stem cells alive and in their undifferentiated state (Dormady *et al.*, 2000; Li *et al.*, 1996; Matsubara *et al.*, 1996; Nakamura *et al.*, 1998; Player *et al.*, 2006).

VIII. OVERLAPPING FUNCTIONS OF GAS6 AND PROTEIN S

From very early after the discovery of the Gas6/Axl ligand/receptor interaction (Varnum *et al.*, 1995), some researchers discovered an interaction of protein S with receptors of the TAM family (Stitt *et al.*, 1995). In this

study, it was proposed that human protein S was able to interact with Tyro3 in a parallel way to Gas6 interaction with Axl. Nevertheless, it was also found that species differences were distinctly important in the affinity of the different ligands with the TAM receptors (Godowski *et al.*, 1995; Mark *et al.*, 1996; Nagata *et al.*, 1996). Indeed, purified human protein S was not able to bind to the extracellular domains of human Tyro3 or the other TAM receptors in the same assays where it was able to bind the extracellular domain of mouse Tyro3. Interestingly enough, bovine protein S seems to be able to bind and activate both the human and mouse receptors (Godowski *et al.*, 1995; Nyberg *et al.*, 1997). Despite this lack of binding activity, human protein S is capable of inducing phosphorylation of human Tyro3 in certain cell types at higher concentrations than human Gas6 or bovine protein S (around 200 nM), but still lower than the concentration of protein S found in plasma (Evenas *et al.*, 2000). In plasma, protein S has a concentration of 350 nM, but almost 60% is bound to the complement regulatory factor C4BP (Giri *et al.*, 2002). Binding to C4BP abolishes protein S binding to TAM receptors (Nyberg *et al.*, 1997), but C4BP does bind Gas6 with a very low affinity (Evenas *et al.*, 1999). One of the problems that makes more difficult to evaluate the results published so far is the capacity of purified protein S of multimerizing in high molecular weight complexes that have specific properties, different from protein S monomers (Heeb *et al.*, 2006). This effect has been studied regarding the anticoagulant properties of protein S, but has not been taken into account in relation to their putative effects on receptor binding. Furthermore, purified Gas6 preparations show a similar behavior, and this could affect the interpretation of the results obtained in purified systems (Stenhoff, Hafizi, and Dahlbäck, personal communication).

Despite these discrepancies on the putative effect of protein S on TAM receptors, several studies *in vivo* suggest that protein S, and in particular human protein S, has a function as a ligand of TAM receptors. On one hand, protein S was found to be the serum factor responsible for the stimulation of the phagocytosis/efferocytosis (Anderson *et al.*, 2003). Protein S-depleted serum or antibodies against protein S could block the effect of serum on clearance of apoptotic cells by macrophages. Furthermore, the effect of purified protein S was also specific for apoptotic cells, not affecting other forms of phagocytosis. This finding is indicative that the Glc-dependent interaction with negatively charged phospholipids membranes was necessary for the effect of protein S (Anderson *et al.*, 2003). A second system where the effect of protein S as a TAM ligand has been demonstrated is the retina. MerTK-deficient animals were found to be blind as a result of the complete degeneration of photoreceptors during the first month of life (Collett *et al.*, 2003; Finnemann and Nandrot, 2006; Hall *et al.*, 2001, 2005; Nandrot and Finnemann, 2006; Prasad *et al.*, 2006). Interestingly, a parallel line of research found that the Royal College of Surgeons (RCS) rat,

a decades-old animal model of retinitis pigmentosa, had a mutated MerTK gene (Nandrot *et al.*, 2000), and mutations in the human MerTK gene have been detected in patients with retinitis pigmentosa (Gal *et al.*, 2000). Several lines of evidence suggest that, at least in mice, the protein S/MerTK pair is the most relevant system in the retina, including the presence of protein S in the retina, the lack of phenotype in Gas6-deficient animals (Hall *et al.*, 2005; Prasad *et al.*, 2006), and the ability of mouse protein S to activate MerTK in extracts of retina (Prasad *et al.*, 2006).

In general, a strong suggestion of a role of protein S as a ligand to TAM receptors derives from comparing different knockout models. If Gas6 would be the only and essential ligand of all three TAM receptors, one would expect a similar phenotype of TAM- and Gas6-deficient animals. This is not the case, as Gas6-deficient animals do not have a similar spontaneous phenotype as compared to the TAM triple knockouts (Angelillo-Scherrer *et al.*, 2001; Lu *et al.*, 1999; Yanagita *et al.*, 2002), not showing signs of autoimmune disease, infertility, or seizures. This clearly indicates the presence of alternative mechanisms of TAM receptor activation, and most likely protein S could be a rescue factor of some of these phenotypes in Gas6-deficient animals. Other ligands, as IL-15 or soluble or cell-bound Axl cannot be excluded (Budagian *et al.*, 2005a, b).

IX. THE IMPLICATIONS OF VITAMIN K IN GAS6 FUNCTION

In most studies, the presence of a properly γ -carboxylated Gla module in Gas6 has been found to be essential for Gas6 function. This was evident from the first report describing the interaction of Gas6 with Axl. The authors already noticed that producing Gas6 under warfarin-induced inhibition of vitamin K-dependent γ -carboxylation abolished completely the Axl receptor autophosphorylation (Varnum *et al.*, 1995). This lack of activity of Gas6 produced on warfarin was extended to *in vitro* binding studies, where Gas6 lacking γ -carboxylated residues was unable to bind Axl (Tanabe *et al.*, 1997). This suggested that the Gla domain could affect the binding site of the receptor, although the LG domains in Gas6 seemed to contain the binding site for TAM receptors (Evenas *et al.*, 2000; Fisher *et al.*, 2005; Mark *et al.*, 1996). Gas6 binds to phosphatidylserine-containing phospholipid membranes in an analogous manner to the rest of the proteins of the family, dependent on the Gla module and therefore of vitamin K action (Hasanbasic *et al.*, 2005; Ishimoto *et al.*, 2000; Nakano *et al.*, 1997a). Warfarin abrogates Gas6-mediated protection of fibroblasts, VSMCs and endothelial cells from serum starvation-induced apoptosis (Hasanbasic *et al.*, 2005; Konishi *et al.*, 2004; Nakano *et al.*, 1997b; Stenhoff *et al.*, 2004).

Proliferation of certain cells could also be abolished by warfarin through a Gas6-dependent mechanism, as in neointima formation (Konishi *et al.*, 2004) and mesangial cell proliferation (Yanagita *et al.*, 1999, 2001a). Finally, macrophage-mediated clearance of apoptotic cells is also inhibited by warfarin effect on Gas6 Gla domain (Hall *et al.*, 2002).

These cellular effects have been translated to *in vivo* models, using oral anticoagulation, to test the effect of inhibition of γ -carboxylation of Gas6 in different contexts. In the retina and other models of efferocytosis, Gas6 needs to be γ -carboxylated in order to be functional (Hall *et al.*, 2002; Ishimoto *et al.*, 2000). Mesangial cell proliferation is also inhibited by warfarin through its action on Gas6 (Yanagita *et al.*, 2001a). The concentration of oral anticoagulants as warfarin necessary to inhibit Gas6-dependent effects were comparable or lower than therapeutic concentrations, indicating that the effects observed in animal models could translate to effects in humans (Yanagita *et al.*, 2001a). Interestingly, Gas6 in plasma has been shown to be affected by oral anticoagulants (Balogh *et al.*, 2005). The decrease in Gas6 concentration in anticoagulated patients compared to a control group was similar to that observed in protein S (Zöller *et al.*, 1995). Although the concentration of Gas6 in plasma could not be as crucial as the local production of the ligand, it could still reflect a systemic effect of oral anticoagulants on the Gas6/Axl system. Therefore, it would be interesting to evaluate if patients in chronic anticoagulant therapy could present side effects derived from the decrease in Gas6 function.

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VITAMIN K₂-MEDIATED APOPTOSIS IN CANCER CELLS: ROLE OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

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Abstract

Vitamin K₂ induces differentiation and apoptosis in a wide array of human cancer cell lines. Vitamin K₂-mediated apoptosis proceeds much more slowly than the apoptosis induced by conventional anticancer agents. Thus, it is possible to analyze the underlying mechanism in detail. In this chapter, we focus on the pro-apoptotic effects of vitamin K₂ on mitochondrial physiology with particular emphasis on changes in mitochondrial membrane potential ($\Delta\Psi m$). Upon treatment of ovarian cancer TYK-nu cells with vitamin K₂, superoxide is produced after two to three days, followed shortly thereafter by release of mitochondrial cytochrome *c*. This is accompanied by other apoptotic features such as characteristic morphological changes and DNA fragmentation by

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day four. Data suggest that superoxide production might cause damage to mitochondrial membranes, open permeability transition pores, and result in disruption of $\Delta\Psi_m$ with subsequent release of cytochrome *c*. Both vitamin K₂-induced production of superoxide and reduction of $\Delta\Psi_m$ are completely inhibited by α -tocopherol such that cell viability is retained. Thus, we propose that the loss of $\Delta\Psi_m$ caused by superoxide might be the major cause of apoptosis following exposure to vitamin K₂. However, other pathways may be involved since cyclosporin A failed to completely inhibit vitamin K₂-induced apoptosis. © 2008 Elsevier Inc.

I. INTRODUCTION

Natural forms of vitamin K, such as vitamin K₁ and vitamin K₂, are essential for blood coagulation, and are used clinically for that purpose. Among various analogs of vitamin K₂ with an unsaturated side chain of 4–13 isoprene units, a form with a geranylgeranyl group (menaquinone-4) is used clinically as an activator of homeostasis (Conly and Stein, 1992) and an inhibitor of osteoporosis with no obvious adverse effects (Shiraki *et al.*, 2000). A novel activity of vitamin K₂, separate from its role in blood coagulation, namely, the induction of differentiation of cultured human leukemia cells, was discovered by Sakai *et al.* (1994). Akiyama *et al.* (1994) first reported the growth-inhibitory effect of vitamin K₂ on mouse osteoclast-like cells. Later, the inhibition of human hepatoma cell line (Hep3B) growth by vitamin K₂ was reported by both Nishikawa *et al.* (1995) and Wang *et al.* (1995). Induction of apoptosis in osteoclasts and primary cultured leukemia cells was demonstrated by Kameda *et al.* (1996) and Yaguchi *et al.* (1997). We showed that vitamin K₂ selectively induces apoptosis in human ovarian cancer TYK-nu cells and pancreatic cancer MiaPaCa-2 cells (Shibayama-Imazu *et al.*, 2003). A characteristic feature of vitamin K₂-induced apoptosis is the slow rate at which it proceeds compared to that by conventional anticancer agents such as camptothecin and etoposide, and by geranylgeraniol (Masuda *et al.*, 2000; Shibayama-Imazu *et al.*, 2003). This feature facilitates the analysis of the underlying mechanism of apoptosis.

It is now well accepted that various apoptosis-inducing signals converge at the mitochondria and release pro-apoptotic proteins such as cytochrome *c*, resulting in the activation of effector caspases which brings about most of the biochemical and morphological alterations associated with apoptosis (Green and Reed, 1998). In the present chapter, we will focus on the novel activity of vitamin K₂ and describe the mechanism of induction of apoptosis. In brief, it appears that production of superoxide damages mitochondrial membranes and triggers the apoptotic cascade.

II. GROWTH-INHIBITORY EFFECT OF VITAMIN K₂

Growth of the Hep3B is inhibited by vitamin K₂ with an IC₅₀ value of ~110 μ M (Bouzahzah *et al.*, 1995; Nishikawa *et al.*, 1995; Wang *et al.*, 1995). Vitamin K₁ does not arrest growth of Hep3B cells, whereas vitamin K₃ potently inhibits growth and induces cell death with an IC₅₀ of 10 μ M (Nishikawa *et al.*, 1995). However, vitamin K₃ subjects both normal and cancer cells to oxidative stress and successful clinical application of vitamin K₃ for cancer patients has not yet been reported. Bouzahzah *et al.* (1995) observed the upregulation of c-myc and c-jun during growth inhibition of hepatoma Hep3B cells by vitamin K₂, although the physiological roles of the expression of the two genes on the vitamin K₂-mediated growth inhibition are not clear. Schneider *et al.* (1998) cloned a set of genes that is strongly expressed in NIH3T3 mouse fibroblasts during growth-arrest due to serum starvation. Manfioletti *et al.* (1993) demonstrated that a protein encoded by a growth-arrest-specific gene, *gas 6*, is vitamin K-dependent. Recently, Hitomi *et al.* (2005) examined the antitumor effects of vitamin K₁, K₂, and K₃ on human hepatocellular carcinoma PLC/PFR/5 cells and reported that vitamins K₂ and K₃ markedly suppressed the growth of these cells, while vitamin K₁ had no effect. They also showed that both vitamins K₂ and K₃ induced G1 arrest via downregulation of expression of cyclin D1 and cyclin-dependent kinase 4.

III. INDUCTION OF DIFFERENTIATION OF LEUKEMIA CELLS BY VITAMIN K₂

Cell-growth arrest is the first step in the induction of differentiation and apoptosis. Sakai *et al.* (1994) demonstrated that human myeloblastic ML1 cells, histiocytic U937, and promyelocytic HL-60 cells differentiate to monocyte-like cells after four days of treatment with vitamin K₂. These effects were not observed with vitamin K₁, which has a phytol side chain. These results suggested that the geranylgeraniol side chain of vitamin K₂ might be essential to induce differentiation. Miyazawa *et al.* (2001) studied the dichotomous nature of vitamin K₂, that is, its capacity to induce both apoptosis and differentiation in leukemic cell lines. Using an HL-60 construct that over-expressed Bcl-2, they demonstrated that the cells were resistant to vitamin K₂-induced apoptosis but differentiated to monocytes in the presence of 10 μ M vitamin K₂. Importantly, control HL-60-neo cells were sensitive to both growth inhibition and apoptosis at equivalent concentrations of vitamin K₂. They concluded that vitamin K₂ induced differentiation of leukemia cells which were otherwise resistant to vitamin K₂-inducing apoptosis.



IV. INDUCTION OF APOPTOSIS BY VITAMIN K₂ IN HUMAN CANCER CELLS

A. Human cancer cell lines sensitive to vitamin K₂

Some inducers of differentiation, such as camptothecin and VP-16, induce apoptosis in cancer cells when their concentrations are higher than those required for induction of differentiation (Barry *et al.*, 1990; Bertrand *et al.*, 1993; Jing *et al.*, 1999; Kaufmann, 1989; Ohnishi *et al.*, 1993). Yaguchi *et al.* (1997) reported that vitamin K₂ analogs such as menaquinone-3, -4, and -5, but not K₁, effectively induced apoptosis in various types of primary cultured leukemia cells and leukemia cell lines *in vitro* at a concentration of 1 μ M. However, compared to vitamin K₂, geranylgeraniol and geranyl-farnesol at equivalent concentrations were less potent at inducing apoptosis in these leukemic cells. Since both vitamins K₁ and K₂ share the naphthoquinone nucleus but only vitamin K₂ induces apoptosis, these results suggest that both the naphthoquinone nucleus and the geranylgeranyl side chain are required for the induction of apoptosis. Vitamin K₂ also induces apoptosis in blastic cells from patients with myelodysplastic syndrome (MDS) (Yaguchi *et al.*, 1998). Takami *et al.* (1999) successfully used vitamin K₂ in the clinical treatment of a patient with this disorder. As shown in Table 10.1, increasing numbers of cancer cell lines have been reported to undergo apoptosis following treatment with vitamin K₂. Recently, Mizuta *et al.* (2006) demonstrated that oral administration of vitamin K₂ had a suppressive effect on the recurrence of hepatocellular carcinoma and improved the patients' survival rates.

B. Mechanism of induction of apoptosis by vitamin K₂

Mitochondria play a central role in controlling cell death and longevity (Hajnocyk and Hoek, 2007). In human MDS, vitamin K₂ induces apoptosis with depolarization of mitochondrial membranes, activation of caspase 3, and DNA fragmentation (Nishimaki *et al.*, 1999; Miyazawa *et al.*, 2001). We reported that vitamin K₂ induced apoptosis in ovarian cancer TYK-nu cells with the reduction of $\Delta\Psi_m$, activation of caspase 3, and release of cytochrome *c* into the cytosol, and DNA fragmentation (Shibayama-Imazu *et al.*, 2003, 2006). These results suggested that vitamin K₂ induces apoptosis through one or more mitochondrial pathways (Green and Reed, 1998).

An increasing body of evidence indicates that the exposure of cells to apoptotic stimuli such as nerve growth factor (NGF) deprivation (Greenlund *et al.*, 1995), vitamin K₃ (Jones *et al.*, 2000), arsenic trioxide (Chen *et al.*, 1998; Iwama *et al.*, 2001), and TNF- α (Moreno-Manzano *et al.*, 2000) lead to the generation of reactive oxygen species (ROS), such as

Table 10.1 Cancer cell lines sensitive to vitamin K₂

Source	Cell line	Reference
Leukemia	ML1	Sakai <i>et al.</i> (1994)
	U937	Sakai <i>et al.</i> (1994)
	HL-60	Sakai <i>et al.</i> (1994); Miyazawa <i>et al.</i> (2001)
	NB4	Yaguchi <i>et al.</i> (1997)
	MDS92	Yaguchi <i>et al.</i> (1998)
	MDS-KZ	Nishimaki <i>et al.</i> (1999)
	KMS-34	Tsujioka <i>et al.</i> (2006)
Ovarian cancer	TYK-nu	Shibayama-Imazu <i>et al.</i> (2003, 2006)
	PA-1	Shibayama-Imazu <i>et al.</i> (in press)
Liver cancer	Hep3B	Wang <i>et al.</i> (1995); Nishikawa <i>et al.</i> (1995); Bouzahzah <i>et al.</i> (1995)
	HepG2	Otsuka <i>et al.</i> (2004)
	PLC/PFR/5	Hitomi <i>et al.</i> (2005)
	HuH-7	Kanamori <i>et al.</i> (2007)
	MiaPaCa-2	Shibayama-Imazu <i>et al.</i> (2003)
Pancreatic cancer		
Lung cancer	LU-139	Yoshida <i>et al.</i> (2003)
Gastric cancer	FU97	Tokita <i>et al.</i> (2006)

superoxide and hydrogen peroxide (H₂O₂), which are thought to be involved in the induction of apoptosis. When human ovarian cancer TYK-nu cells were treated with vitamin K₂ for two to three days, superoxide was readily detectable (Fig. 10.1). During the first two days of treatment with vitamin K₂, apoptotic processes induced in TYK-nu cells were reversible. However, by the third day, the process became irreversible after the production of superoxide (Fig. 10.1 and see also Shibayama-Imazu *et al.*, 2003). Interestingly, TYK-nu cells released cytochrome *c* from mitochondria into the cytosol after three days of treatment with vitamin K₂ (Fig. 10.1). Other apoptotic features such as characteristic morphological changes and DNA fragmentation were not observed during the initial 3-day period in the vitamin K₂-treated cells but became apparent on day 4 (Fig. 10.1).

There are several potential cellular sources for the production of ROS, including cytosolic xanthine oxidase, plasma-membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lysosomes, peroxisomes, the endoplasmic reticulum, and the mitochondrial electron-transport system (Moldovan and Moldovan, 2004; Turrens, 1997). Vitamin K₂-induced production of superoxide in TYK-nu cells was unaffected by allopurinol, an inhibitor of superoxide production by cytosolic xanthine oxidase (Massey *et al.*, 1970). In addition, diphenyleneiododinium chloride

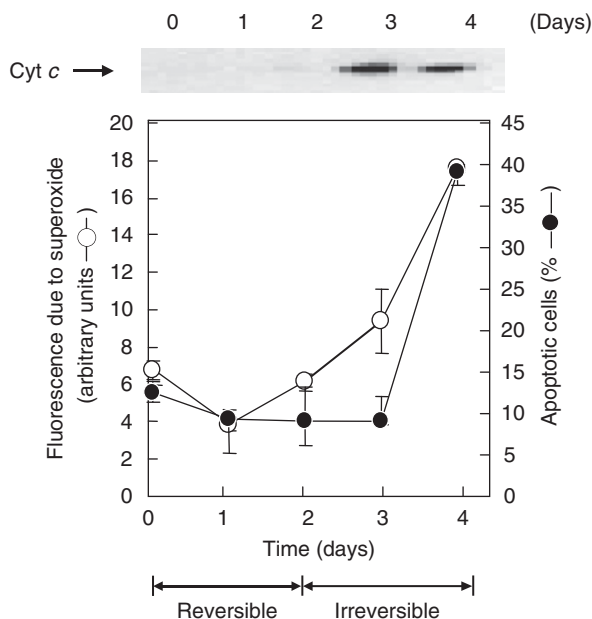


Figure 10.1 Processes of the induction of apoptosis by treatment of human ovarian cancer TYK-nu cells with vitamin K₂ (VK₂). TYK-nu cells were treated with 150 μ M VK₂. Production of superoxide (○), induction of apoptosis (●), and release of cytochrome c from mitochondria were measured as described previously (Shibayama-Imazu *et al.*, 2003). Data are expressed as means $\pm$ SD from four independent experiments. [Reproduced by permission from Fig. 3 of Shibayama-Imazu *et al.* (2006). Copyright © 2006 Springer Science + Business Media.]

(DPI), an inhibitor of membrane NADPH oxidase, had no effect on vitamin K₂-induced production of superoxide in TYK-nu cells (Cross, 1990; Kojima *et al.*, 1990). These results suggest that mitochondria might be the major site of production of superoxide in vitamin K₂-treated TYK-nu cells. Although cellular H₂O₂ plays a key role in the induction of apoptosis in some cell types (Chen *et al.*, 1998; De Bleser *et al.*, 1999), vitamin K₂-induced apoptosis in TYK-nu cells was not affected by the addition of 400 units/ml catalase (a scavenger of H₂O₂), or 400 units/ml superoxide dismutase (SOD), which catalyzes the production of H₂O₂ from superoxide (data not shown). These results suggested that the amount of H₂O₂ produced in TYK-nu cells by vitamin K₂ treatment might have been too low to contribute to apoptotic signaling.

α -Tocopherol is a lipophilic antioxidant molecule and suitable for scavenging free radicals such as superoxide and the peroxy, hydroxyl, and hydroperoxy radicals in a hydrophobic environment as in cellular membranes (Halliwell and Gutteridge, 1998). α -Tocopherol completely inhibits the production of superoxide in vitamin K₂-treated TYK-nu cells and

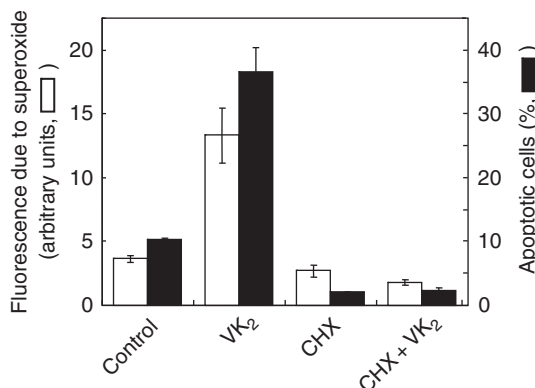


Figure 10.2 Effects of cycloheximide (CHX) on induction of apoptosis and production of superoxide during treatment of TYK-nu cells with vitamin K₂ (VK₂). TYK-nu cells were treated with 150 μ M VK₂ in the absence or presence of CHX (100 ng/ml for four days). Data are expressed as means $\pm$ SD from two independent experiments. The methods for measurement of production of superoxide (white columns) and induction of apoptosis (black columns) were described previously (Shibayama-Imazu *et al.*, 2003). [Reproduced by permission from Fig. 4 of Shibayama-Imazu *et al.* (2006). Copyright © 2006 Springer Science + Business Media.]

efficiently blocks vitamin K₂-induced apoptosis (Shibayama-Imazu *et al.*, 2006). These results suggest that most of the superoxide associated with the induction of apoptosis by vitamin K₂ might be produced in mitochondrial membranes.

As shown in Fig. 10.2, both the production of superoxide and the induction of apoptosis by vitamin K₂ were almost completely inhibited by cycloheximide (CHX), an inhibitor of protein synthesis, suggesting that *de novo* synthesis of enzymes for the production of superoxide might be required for these processes (Shibayama-Imazu *et al.*, 2006).

V. ASSOCIATION WITH REDUCED MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_m$)

There are many reports suggesting that both the generation of superoxide and a reduction in $\Delta\Psi_m$ might be associated with the induction of apoptosis (Fariss *et al.*, 2005; Kowaltowski and Vercesi, 1999). The mitochondrial inner membrane has a $\Delta\Psi_m$ value of -150 mV, which results from both the proton gradient and the difference of charges between inner membrane and mitochondrial matrix. The proton gradient is generated by the transportation of protons through electron transporter complexes I, III, and IV in the respiratory chain of mitochondria (Fariss *et al.*, 2005;

Lenaz, 2001). The maintenance of a proper $\Delta\Psi_m$ is essential for the survival of the cell as it drives the synthesis of ATP and maintains oxidative phosphorylation. (Gottlieb, 2001). Using a $\Delta\Psi_m$ detection probe (JC-1) and a flow cytometric approach, Miyazawa *et al.* (2001) found that vitamin K₂ induces depolarization of $\Delta\Psi_m$ in HL-60 cells. Recently, Tsujioka *et al.* (2006) examined the human myeloma cell line KMS-34 with flow cytometry using DiOC6 and found that vitamin K₂ induced changes in $\Delta\Psi_m$.

In order to monitor depolarization of mitochondrial membranes, we used MitoTracker Red CMXRos (CMXRos), a positively charged rhodamine derivative which is rapidly taken up by negatively charged (functioning) mitochondria. Therefore, its loading is expected to be dependent on $\Delta\Psi_m$ and mitochondrion-specific. When $\Delta\Psi_m$ is reduced and mitochondria lose their negative charge, CMXRos is no longer incorporated into mitochondria, suggesting that function has been lost. Furthermore, this dye contains a thiol-reactive moiety, which is responsible for keeping the dye associated with the mitochondria after fixation (Poot *et al.*, 1996). This characteristic is a great advantage for analyzing $\Delta\Psi_m$ after fixation of mitochondria with formaldehyde for fluorescent microscopy. When TYK-nu cells were treated with vitamin K₂ for 96h, a smaller amount of CMXRos accumulated in mitochondria (Photograph 2 in Fig. 10.3A and column 2 in Fig. 10.3B), suggesting that the induction of apoptosis by vitamin K₂ might involve a reduction in $\Delta\Psi_m$ (Shibayama-Imazu *et al.*, 2006). Although the fluorochromes used for detecting the induction of apoptosis were different in these three reports described above, it is obvious that vitamin K₂ induces a drop in $\Delta\Psi_m$ during induction of apoptosis.

The permeability transition pore (PTP) consists mainly of the mitochondrial outer membrane voltage-dependent anion channel (VDAC), inner membrane adenine nucleotide translocase (ANT), and the mitochondrial benzodiazepine receptor (cyclophilin D) (Crompton, 1999; Shimizu *et al.*, 2001). The probability of pore opening increases with a decrease in $\Delta\Psi_m$ and decreases with acidification below pH 7 of the matrix (Bernardi *et al.* 1992). When PTPs open, solutes, such as K⁺, Mg²⁺, and Ca²⁺ ions, and water, enter into mitochondria, leading to swelling of the mitochondrial matrix, rupturing of the outer membrane, and eventual leakage of mitochondrial proteins such as cytochrome *c* and apoptosis-inducing factor (Kroemer, 1997). Potent inducers of PTP opening such as Ca²⁺ and tert-butylhydroperoxide are also potent inducers of apoptosis (Hatanaka *et al.*, 1996; Klaidman *et al.*, 1996; Takei and Endo, 1994). The opening of PTPs has been demonstrated to reduce $\Delta\Psi_m$, followed by the release of cytochrome *c* (Crompton, 1999; Ly *et al.*, 2003; Shimizu *et al.*, 2001; Yang and Cortopassi, 1998). However, accumulating evidences suggest that apoptosis can be induced without significant changes in $\Delta\Psi_m$ and that the loss of $\Delta\Psi_m$ could be detected only later in the cell death pathway (Cossarizza

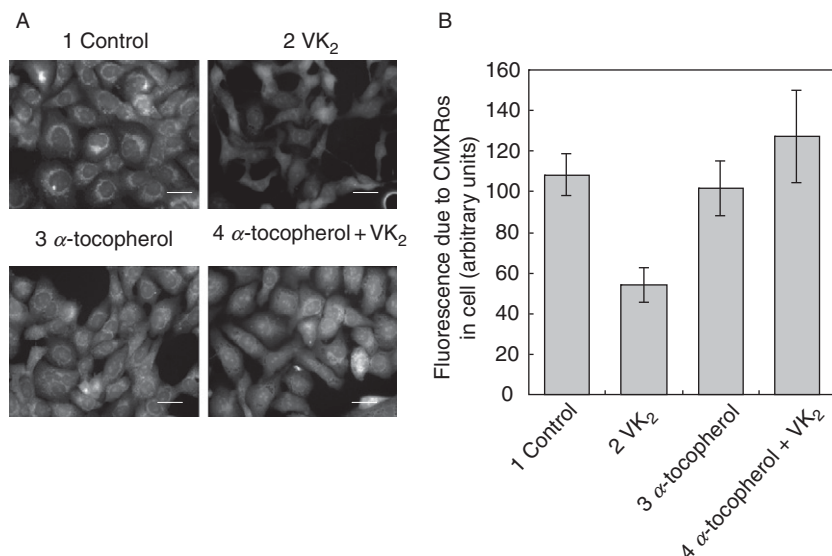


Figure 10.3 Effects of α -tocopherol on the vitamin K₂ (VK₂)-induced reduction of mitochondrial transmembrane potential. TYK-nu cells were treated with 150 μ M VK₂ in the absence or presence of 50 μ M of α -tocopherol. (A) After incubation for four days, MitoTracker Red CMXRos was added at 400 nM to the culture medium and incubation was continued for a further 15 min. Cells were fixed in 4% formaldehyde, washed with phosphate-buffered saline (PBS) and mounted. Photographs were taken under a fluorescence microscope. Representative results from three experiments are shown. Bars represent 25 μ m. (B) The fluorescence of cells in pictures shown was represented as numerical values and average values from eight cells were presented. [Partly reproduced by permission from Fig. 7 of Shibayama-Imazu *et al.* (2006). Copyright © 2006 Springer Science + Business Media.]

et al., 1994; Finucane *et al.*, 2002; Karpinich *et al.*, 2002; Ly *et al.*, 2003; Nopp *et al.*, 2002). It is, therefore, likely that the reduction of $\Delta\Psi_m$ is required by some (but not all) cell types and apoptosis inducers. Once cytochrome *c* is released from mitochondria into the cytoplasm, it combines with cytosolic factors such as dATP and apoptotic protease activating factor-1 (Apaf-1), leading to activation of caspase 9, which, in turn, activates caspase 3 (Li *et al.*, 1997; Zou *et al.*, 1999).

As shown by Photograph 4 in Fig. 10.3A and column 4 in Fig. 10.3B, the reduction in $\Delta\Psi_m$ in response to vitamin K₂ was almost completely inhibited by α -tocopherol. Furthermore, α -tocopherol inhibited the induction of apoptosis most effectively when it was added two days after the start of incubation with vitamin K₂ (Shibayama-Imazu *et al.*, 2006). Thus, early addition of α -tocopherol inhibited the vitamin K₂-induced reduction of $\Delta\Psi_m$ and apoptosis. Since α -tocopherol reacts with oxygen radicals, such as superoxide, within the hydrophobic environment of the mitochondrial

membrane, we postulate that vitamin K₂-induced superoxide in mitochondria might damage mitochondrial membranes, with subsequent release of cytochrome *c*, the activation of procaspase 3, and finally result in induction of apoptosis (Fig. 10.4).

Mitochondrial release of cytochrome *c* appears to occur in one of two ways: (1) by the opening of mitochondrial PTPs and the reduction of $\Delta\Psi_m$ (Scarlett and Murphy, 1997, Shimizu *et al.*, 2001) or (2) by the action of pro-apoptotic members of the Bcl-2 family of proteins, such as Bid, Bax, and Bak, which interact with PTPs (Danial and Korsmeyer, 2004; Narita *et al.*, 1998). We tested whether cyclosporin A, which inhibits permeability transition by binding to cyclophilin D (Broekemeier *et al.*, 1989), might inhibit the induction of apoptosis by vitamin K₂. We found that cyclosporin A inhibited vitamin K₂-mediated apoptosis of TYK-nu cells by ~50% (data not shown). This suggests that the vitamin K₂-induced apoptosis is not exclusively regulated by permeability transition. On the other hand, it is also possible that cytochrome *c* is released through from Bax-Bax channels,

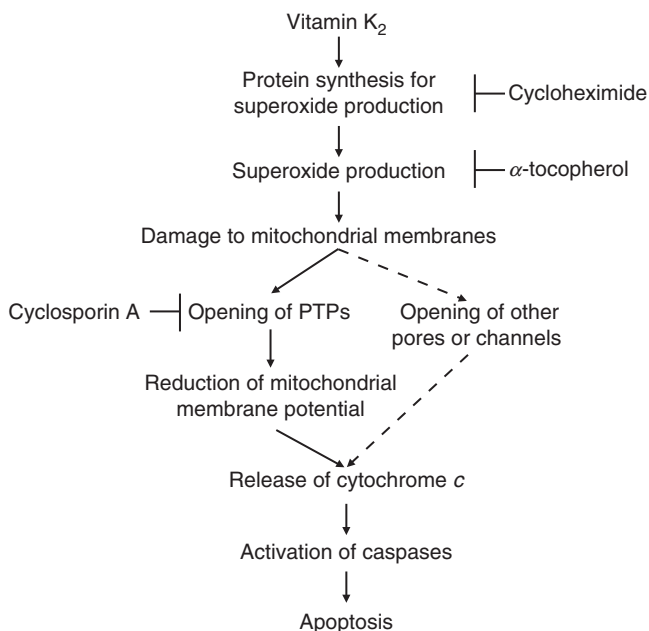


Figure 10.4 Schematic representation of the mechanism of induction of apoptosis by vitamin K₂ (VK₂) in ovarian cancer TYK-nu cells. The major pathway for the induction of apoptosis by VK₂ is via opening of permeability transition pore (PTP) by superoxide formed by the VK₂ treatment, with subsequent reduction of mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome *c*. Opening of other pores or channels also may contribute to the induction of apoptosis. Induction of apoptosis by VK₂ is completely inhibited either by cycloheximide or by α-tocopherol, but inhibition of VK₂-induced apoptosis by cyclosporine A is not complete.

Bax/Bid-lipid pores, and a Bax-VDAC channels (Antonsson *et al.*, 2000; Korsmeyer *et al.*, 2000; Marzo *et al.*, 1998; Saito *et al.*, 2000; Shimizu *et al.*, 2000). In fact, we detected the accumulation of Bax in a mitochondrial fraction when cells were treated with vitamin K₂ for 72 h (data not shown).

Mitochondria are particularly susceptible to ROS (Fariss *et al.*, 2005; Turrens, 1997), which irreversibly damage mitochondrial DNA, membrane lipids, and proteins, with resultant mitochondrial dysfunction and, ultimately, cell death (Kowaltowski and Vercesi, 1999). Marshansky *et al.* (1983) demonstrated that peroxidation of unsaturated fatty acids in mitochondria induces swelling of mitochondria, and efflux of potassium ions, with simultaneous decrease in $\Delta\Psi_m$. Recent studies have shown that the peroxidation by ROS of the acidic phospholipid cardiolipin, which is abundant in the inner leaflet of the mitochondrial membrane, induces the release of cytochrome *c* from mitochondria into the cytosol (Nomura *et al.*, 2000; Petrosillo *et al.*, 2003). An increasing body of evidence also indicates that cytochrome *c* binds specifically to cardiolipin and that peroxidation of cardiolipin induces the release of cytochrome *c* (Kagan *et al.*, 2004; Nomura *et al.*, 2000; Petrosillo *et al.*, 2003; Shidoji *et al.*, 1999; Touminen *et al.*, 2002). Peroxidation of cardiolipin also causes dysfunction or structural change of ANT (Giron-Calle *et al.*, 1994), which is present in inner mitochondrial membranes and binds electrostatically to six surrounding cardiolipins (Beyer and Klingenberg, 1985), leading to induction of permeability transition and opening of PTP (Imai *et al.*, 2003). Thus, it is possible that the superoxide formed in TYK-nu cells in response to vitamin K₂ might contribute to the release of cytochrome *c* from mitochondria via the peroxidation of cardiolipin.

VI. CONCLUSION

Vitamin K₂ induces apoptosis in the human cancer cell lines as listed in Table 10.1. Vitamin K₂-mediated apoptosis occurs slowly, making it possible to analyze its mechanism in detail. Upon treatment of ovarian cancer TYK-nu cells with vitamin K₂, protein(s) required for production of superoxide is synthesized. The superoxide produced might cause damage to mitochondrial membranes, open PTPs, and result in disruption of $\Delta\Psi_m$, with subsequent release of cytochrome *c* (Fig. 10.4). Both vitamin K₂-induced production of superoxide and the reduction of $\Delta\Psi_m$ are completely inhibited by the antioxidant α -tocopherol. Thus, we conclude that the loss of $\Delta\Psi_m$ by superoxide might be the major pathway for the induction of apoptosis by vitamin K₂. Peroxidation of cardiolipin by superoxide formed by vitamin K₂-treatment might contribute to the release of cytochrome *c* by altering the interaction between cardiolipin and cytochrome *c* and by opening

of PTPs. However, incomplete inhibition of vitamin K₂-induced apoptosis by cyclosporin A suggests the contribution of other pathways such as opening of other channels.

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VKORC1: A WARFARIN-SENSITIVE ENZYME IN VITAMIN K METABOLISM AND BIOSYNTHESIS OF VITAMIN K-DEPENDENT BLOOD COAGULATION FACTORS

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Abstract

The recently discovered enzyme VKORC1 of the vitamin K cycle, which is the target for the anticoagulant drug warfarin, has opened new opportunities to understand warfarin resistance and biosynthesis of vitamin K-dependent blood coagulation factors and other members of this protein family. Furthermore, it has opened new opportunities to study the vitamin K-dependent posttranslational γ -carboxylational system in the endoplasmic reticulum in greater detail and its molecular operation *in vivo*. Other accomplishments resulting from this discovery are: (1) the finding that VKORC1 is the rate-limiting step in biosynthesis of functional vitamin K-dependent proteins, and (2) engineering of recombinant intracellular γ -carboxylation systems in cell lines producing

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recombinant coagulation factor used clinically to treat bleeding disorders. The engineered cells significantly enhance production of the fraction of fully functional γ -carboxylated proteins compared to cell lines only overexpressing the specific coagulation factor. The first described inhibitor of the γ -carboxylation system has been identified as calumenin, a resident chaperone in the endoplasmic reticulum (ER). Together, the new information gained about the vitamin K-dependent γ -carboxylation system will stimulate new research which will benefit medicine and our understanding of the molecular mechanisms involved in this protein modification reaction. © 2008 Elsevier Inc.

I. INTRODUCTION

A. Discovery of vitamin K

The discovery of vitamin K resulted from a series of experiments carried out by Henrik Dam on the possible essentiality of cholesterol in the diet of the chick (Dam, 1929a, b, 1930). He noted that chicks exposed to food that had been extracted with nonpolar solvents to remove sterols, developed subdural and muscular hemorrhages and that blood taken from these animals clotted slowly. He made the important observation that adding cholesterol to the diet did not correct the bleeding problem. Based on these observations, he concluded that a nonpolar component responsible for the hemorrhage was removed from the diet by the extraction, and focused his research on identifying this component. The disease was subsequently observed by McFarlane *et al.* (1931) who described a clotting defect seen when chicks were fed ether-extracted fish or meat meal in an attempt to establish the vitamin A and D requirements of the chick. However, Dam (1935a, b) proposed that the antihemorrhagic factor was a “new” vitamin which he named vitamin K (K from the first letter in the German word “Koagulation”). His reported discovery of a “new” vitamin was followed by independent reports by Almquist and Stockstad (1935a, b) describing their success in curing the hemorrhagic disease with ether extracts from alfalfa. Later on, Quick and Stefanini (1949) recognized that the clotting defect in the chicks fed the hemorrhagic diet resulted from the lack of the coagulation factor, prothrombin. Since an increased blood concentration of prothrombin did not cure the defect, it is understandable that many years went by before the mechanism underlying the bleeding etiology was understood. The discovery that factor VII (Owen *et al.*, 1951), factor X, (Hougie *et al.*, 1957), and factor IX (Aggeler *et al.*, 1952; Biggs *et al.*, 1952) were dependent on vitamin K for their synthesis strongly suggested that vitamin K was involved in synthesis of certain clotting factors of the blood coagulation system. Dam in collaboration with Karrer at the University of Zurich succeeded in 1939 in the isolation of vitamin K₁ as an oil from alfalfa

(Dam *et al.*, 1939). At the same time, other research groups also isolated vitamin K from alfalfa and putrefied fish meal (Karrer and Geiger, 1939; McKee *et al.*, 1939). Doisy's group (MacCorquodale *et al.*, 1939) characterized vitamin K₁ from alfalfa as 2-methyl-3-phytyl-1,4-naphthoquinone and later the same group (Binkley *et al.*, 1940) characterized the vitamin K component from fish meal as a vitamin K derivative with an unsaturated side chain which the group named vitamin K₂. For their achievements, Dam and Doisy shared the Nobel Prize in Physiology and Medicine in 1943. Vitamin K is made by plants and bacteria. Today we know that the plant vitamin, phyloquinone or vitamin K₁, is a naphthoquinone with a 17-carbon isoprenoid side chain linked to the 3 position on the naphthoquinone ring system (Fig. 11.1). Menaquinones (MK-*n*), or vitamin K₂s, are made by bacteria. They constitute a series of vitamin K₂s with an unsaturated isoprenoid side chain in the 3 position of various lengths.

B. Discovery of dicumarol and synthesis of warfarin

During the period that Dam was involved in establishing the antihemorrhagic function of vitamin K, the first antagonist to the action of vitamin K was being investigated as a compound in spoiled sweet clover hay consumed by cattle in Canada and the American Midwest in the 1920s which caused severe bleeding in the cattle (Link, 1959). The compound was isolated by Link's group (Cambell and Link, 1941; Stahmann *et al.*, 1941) and was characterized as 3,3'-methyl-bis-4-hydroxycoumarin.

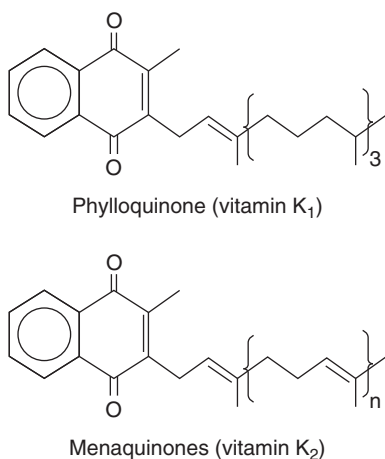


Figure 11.1 The fat-soluble vitamin K family. Phylloquinone (vitamin K₁) is made by plants and the vitamin has a saturated isoprenoid side chain of 17 carbon atoms. Menaquinones are a group of vitamin Ks made by bacteria, each having an unsaturated isoprenoid side chain of variable length.

The compound was given the name “dicumarol” (Fig. 11.2). Dicumarol was used clinically as an anticoagulant to treat thromboembolic disease but its liver toxicity triggered Link’s group to chemically modify dicumarol to a compound with less toxicity. The most successful compound was called warfarin which today is an extensively used anticoagulant in long-term treatment of thromboembolic disease.

C. Discovery of the vitamin K modification and vitamin K-dependent proteins

A race between Dr. Stenflo’s group in Sweden (Stenflo *et al.*, 1974) and Dr. Nelsestuen’s group (Nelsestuen *et al.*, 1974) in the United States in the early 1970s resulted in discovery of the role vitamin K plays in biosynthesis of the vitamin K-dependent blood clotting factors. By comparing the structure of prothrombin isolated from warfarin-treated bovine plasma and normal bovine plasma, the researchers identified a “new” amino acid in normal prothrombin which was absent in prothrombin isolated from cows treated with the vitamin K antagonist, warfarin. The new amino acid residue in normal prothrombin was identified as γ -carboxyglutamic acid (Fig. 11.3).

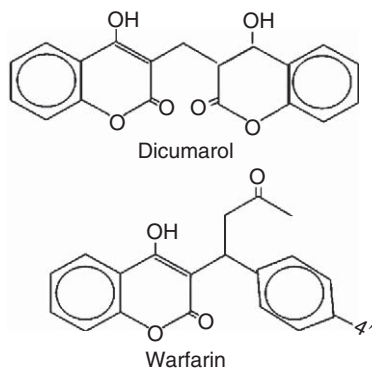


Figure 11.2 The VKORC1 inhibitors, dicumarol and warfarin. The synthetic and clinically used drug, warfarin, is shown with the 4' position in the benzene ring facing away from the active hydroxyl-coumarin ring system.

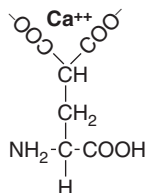


Figure 11.3 γ -Carboxyglutamic acid (Gla) with chelated Ca^{2+} .

It became clear that vitamin K was involved in a posttranslational modification reaction leading to Gla modification of a specific set of Glu residues in newly synthesized precursor proteins of clotting factors II, VII, IX, and X. The Gla residues are Ca^{2+} binding residues (see Fig. 11.3) and the discovery of the Gla modification revealed why Ca^{2+} is essential in blood clotting. Today we know that Ca^{2+} induces a conformational change in the region of the vitamin K-dependent proteins harboring the Gla residues (Gla region) which is essential for the blood clotting factors to bind to phospholipids at the site of injury where they undergo a proteolytic activation process to become active coagulation factors from zymogens (Furie and Furie, 1992).

The family of vitamin K-dependent proteins has expanded significantly since the original findings of these modified proteins in the blood coagulation system. To date, 14 proteins in vertebrates that are known, or strongly suspected, to undergo the γ -carboxylation modification have been identified (see Table 11.1). Several new members of the family are not blood coagulation factors and include matrix Gla protein (MGP), Gas6, protein S (also blood coagulation), and transmembrane receptors which are widely expressed in the body (Kulman *et al.*, 1997, 2001; Loeser *et al.*, 1994; Stenflo and Dahlback, 2001; Price *et al.*, 1998). Osteocalcin is synthesized exclusively in bone cells (Price *et al.*, 1998).

So far, the only invertebrates that have been confirmed at the protein level to synthesize proteins with the vitamin K-dependent Gla modification are marine snails from the gastropode genus, *Conus*. These snails γ -carboxylate toxic neuropeptides (conotoxins) used to immobilize and kill prey (Fainzilber *et al.*, 1998; Hansson *et al.*, 2004; McIntosh *et al.*, 1984; Olivera *et al.*, 1990). The metal binding by the Gla residues in the conotoxins is important for inducing and stabilizing a conformational change required for their specific interaction with neuromuscular receptors and ion channels (Grant *et al.*, 2004). Because of the extensive sequence homology among the Gla region in vertebrate vitamin K-dependent proteins and the existence of completed genomic databases, all members of the vitamin K-dependent protein family in vertebrates are likely to have been identified. On the other hand, the Gla modification is old in evolution and future research may discover new biological functions where the modified Glu residues are involved.

II. THE VITAMIN K-DEPENDENT γ -CARBOXYLATION SYSTEM

Since the success by which all members of the vitamin K-dependent protein family become physiologically active is dependent upon completion of their Gla modification, the posttranslational vitamin K-dependent

Table 11.1 The vitamin K protein and peptide family

Identified or hypothetical vitamin K-dependent protein or peptide	Physiological function of protein or peptide	Species and characteristics of the protein or peptide
Protein S	Blood coagulation	Vertebrates, proenzyme
Protein C	Blood coagulation	Vertebrates, proenzyme
Protein Z	Blood coagulation	Vertebrates, proenzyme
Factor VII	Blood coagulation	Vertebrates, proenzyme
Factor IX	Blood coagulation	Vertebrates, proenzyme
Factor X	Blood coagulation	Vertebrates + snake, proenzyme
Prothrombin	Blood coagulation	Vertebrates + hagfish, proenzyme
Gas6	Blood coagulation (platelets)	Vertebrates, Tyro3-ligand
MGP	Calcification inhibitor	Vertebrates, BMP-2 binding
Osteocalcin	Bone formation	Vertebrates, bone regulation
PRGP1★	?	Vertebrates, membrane protein
PRGP2★	?	Vertebrates, membrane protein
TMG1★	?	Vertebrates, membrane protein
TMG2★	?	Vertebrates, membrane protein
Gla-RTK	?	Tunicates, tyrosine kinase
Conotoxins	Neurotoxin	Gastropodes, muscular blocker

RTK, Gla-RTK, receptor tyrosine kinase; MGP, matrix Gla protein; Gas6, growth arrest-specific protein; TMG, transmembrane Gla protein; PRGP, proline-rich Gla protein. Protein names labeled with ★ have not been confirmed by chemical analysis to contain Gla but the proteins have an homologous Gla region.

γ -carboxylation system plays an important role in homeostasis. Indeed, impairments of functioning of the system in liver are known to cause serious bleeding disorders (Mammen, 1992). The recent finding by Karsenty's

group (Murshed *et al.*, 2004) that MGP synthesized by vascular smooth muscle cells in the arterial wall, and not systemic MGP, is essential for MGP to work as an aortic calcification inhibitor strongly indicates that impairment of extrahepatically located systems can be responsible for pathology in organs and tissues. Therefore, in order to improve our understanding of pathology associated with improper functioning of the proteins belonging to the family of vitamin K-dependent proteins (Table 11.1), it is essential that we understand the “molecular machinery” constituting the system in addition to the genetics of (1) the components of the system as well as (2) the genetics of the proteins becoming modified by the system.

As mentioned in Section I, the vitamin K-dependent Gla modification system also plays an important role in prophylactic medicine. The system is the target for coumarin anticoagulants used in long-term anticoagulation therapy to control thromboembolic disease (Furie and Furie, 1992; Wallin and Hutson, 2004). Warfarin (USA) and acenodicumarol (modified form of warfarin) (Europe) have saved the life of millions of people since warfarin was introduced into medicine in the 1950s (Suttie, 1978).

The vitamin K-dependent γ -carboxylation system is a multicomponent system of proteins embedded in the endoplasmic reticulum (ER) membrane (Furie and Furie, 1992). The system (see Fig. 11.4) consists of: (1) the vitamin K-dependent γ -carboxylase which requires the reduced hydroquinone form of vitamin K (Vit. K_1H_2) as cofactor, and (2) the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which produces the cofactor. Concomitant with γ -carboxylation, the hydroquinone is converted to the metabolite vitamin K 2,3-epoxide (Vit. $K_1 > O$), which is reduced back to the Vit. K_1H_2 cofactor by VKOR. This interconversion of vitamin K metabolites is known as the vitamin K cycle (Suttie, 1978) (see Fig. 11.4). The complete set of reactions shown in Fig. 11.4 constitutes the vitamin K-dependent γ -carboxylation system.

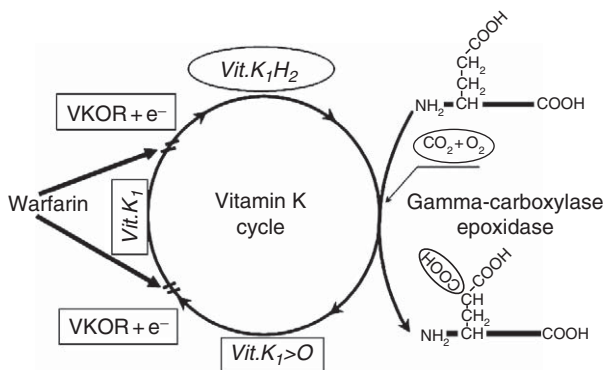


Figure 11.4 The vitamin K-dependent γ -carboxylation system. Warfarin inhibits reduction of Vit. $K_1 > O$ by VKOR to the reduced vitamin K_1H_2 γ -carboxylase cofactor.

The purification of γ -carboxylase from the ER membrane (Wu *et al.*, 1991) resulted in a broad understanding of this membrane enzyme and the mechanism by which the enzyme carries out vitamin K-dependent modification of proteins (Pudota *et al.*, 2001). VKOR, on the other hand, has proven to be extremely difficult to be purified from the ER membrane which has hampered efforts to understand the molecular mechanism by which VKOR produces the reduced vitamin K_1H_2 cofactor. However, recent breakthroughs have been accomplished which have made it possible to study VKOR and thus the vitamin K-dependent γ -carboxylation system in greater detail at the molecular level. These breakthroughs have been (1) the ability of Kohn *et al.* (2003) to map warfarin resistance to the *Rw* locus on chromosome 1 in the rat; (2) the investigation by Fregin *et al.* (2002) of two families with familial multiple coagulation factor deficiency (FMFD), where the patients have a deficiency in all of the vitamin K-dependent coagulation factors which mapped warfarin resistance in man to the 16p12-q21 region on chromosome 16; (3) the work by Rost *et al.* (2004) who sequenced genomic DNA in the 16p12-q21 region and identified the VKOR gene; (4) the work by Li *et al.* (2004) who used a small interfering RNA (siRNA) approach to silence integral membrane proteins encoded by genes in the 16p12-q21 region and arrived at the same finding as Rost *et al.* (2004); (5) the bioinformatic analyses of the VKOR gene carried out by Goodstadt and Ponting (2004) who predicted VKOR to be an 18 kDa highly hydrophobic membrane protein with a thioredoxin CXXC redox center in the third hydrophobic transmembrane domain at the C-terminal side of the protein; and (6) the CXXC mutagenesis work by Wajih *et al.* (2005) on the predicted CXXC center which showed that the CXXC center is the redox center in VKOR. A hydropathy plot of VKOR which predicts three transmembrane domains is shown in Fig. 11.5. The location of the thioredoxin fold with the CXXC redox center is shown in the C-terminal oriented 3rd transmembrane domain.

Early on, numerous attempts by various research groups (Lee *et al.*, 1985; Suttie, 1978; Wallin *et al.*, 2001) to purify VKOR from the ER membrane failed. The difficulties associated with this task became easier to understand after the *VKORC1* gene was identified. Rost *et al.* (2004) named the 18 kDa translated protein, VKORC1, as an abbreviation for vitamin K epoxide reductase complex subunit 1. This abbreviation is now commonly found in the literature and should be associated with the original enzyme vitamin K 2,3-epoxide reductase of the vitamin K cycle, a name that has been used since this warfarin-sensitive enzyme was discovered by Matschiner *et al.* (1970). Chu *et al.* (2006) have partially purified recombinant VKORC1 expressed in insect cells which *in vitro* show warfarin-sensitive vitamin K_1 2,3-epoxide reduction when tested in a system containing a high DTT concentration at an high alkaline pH of 8.5 which has been avoided by research groups in the past because of nonenzymatically

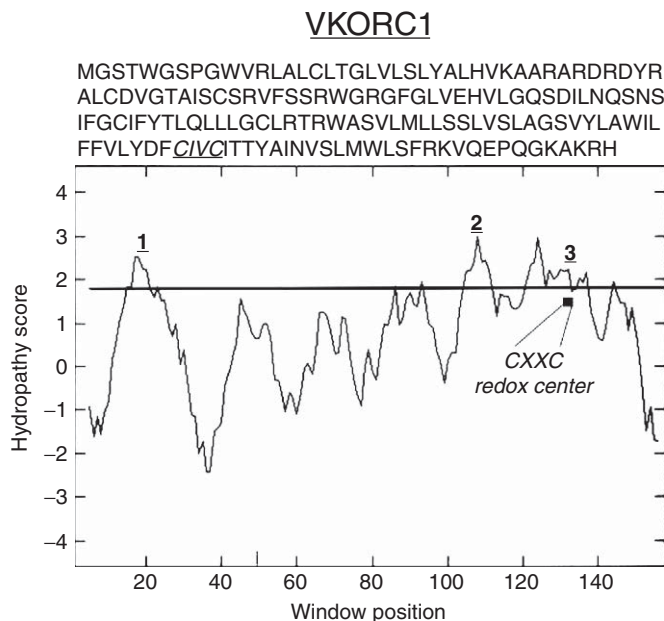


Figure 11.5 Kyte Doolittle hydropathy plot of rat VKORC1. The scoring system predicts three transmembrane domains labeled 1, 2, and 3. The position of the CXXC redox center in the hydrophobic 3 transmembrane domain is shown. The primary sequence of the translated protein is also shown with the redox center in *italic* (CIVC).

catalyzed reduction of the epoxide by DTT at alkaline pH. However, these extreme assay conditions may indicate that reconstitution of recombinant VKORC1 is difficult because of its extreme hydrophobic properties. On the other hand, VKOR activity, when measured in a suspension of extensively washed ER membrane vesicles from rat liver, exhibits very high activity at a significantly lower pH (Wallin *et al.*, 2002). In these assays, VKOR is assembled in a “natural” environment with lipids and other membrane proteins which may indicate that the partially purified recombinant VKORC1 protein is not correctly assembled with other ER components to exhibit maximum activity.

The discoveries by Matschiner *et al.* (1970) of vitamin K₁ 2,3-epoxide as a metabolite of vitamin K₁ in liver and the warfarin-sensitive vitamin K cycle by Whitton *et al.* (1978) led to numerous attempts to characterize VKOR at the biochemical level. From these early studies, it became clear that thiols are involved in the catalytic mechanism of vitamin K₁ 2,3-epoxide reduction by VKOR and suggested that warfarin binds to a thiol redox center in VKOR. In those days, the most plausible model for the mechanism of inhibition of VKOR by warfarin came from work in Dr. Fasco’s laboratory (Fasco *et al.*, 1983). Their experimental data, which

was also confirmed by our laboratory (Wallin *et al.*, 1986), led to the proposal that warfarin binds to an oxidized thiol redox center and prevents reduction of the center by an unknown electron donor. Despite numerous attempts, the identity of the physiological electron donor and the mechanism of reduction of the VKOR thiol redox center were not resolved. Since these early attempts were hampered by unsuccessful attempts to purify VKOR, early research on VKOR by our laboratory was carried out with the goal of obtaining a semi-purified preparation of VKOR where individual proteins of a putative VKOR enzyme complex could be separated by 2D-SDS-PAGE and identified by mass spectrometry (MS/MS). To identify components of VKOR in the semi-purified preparation of VKOR, we used 4'-azido-warfarin-³H-alcohol, a potent VKOR inhibitor, as an affinity label. Two ³H-labeled proteins were identified by MS/MS in the 2-D-SDS-PAGE gels. These proteins were calumenin and cytochrome B₅ (Wallin *et al.*, 2001). The 4'-azido derivative of warfarin did not label VKOR. Based on the expected orientation of the 4'-azido-derivative in its binding site on VKOR (Wallin *et al.*, 2001), this finding was not surprising. The coumarin ring system of the inhibitor would occupy the binding site on VKOR and the 4'-azido group would be expected to attach, by photo activation, to peripheral proteins associated with VKOR and other proteins of the γ -carboxylation system (see Fig. 11.4). Rat calumenin was cloned and sequenced and the recombinant protein produced. When added to an *in vitro* test system, the 47-kDa recombinant protein was found to inhibit VKOR activity and to protect the enzyme from warfarin inhibition. Calumenin was found to be a more potent inhibitor of the complete vitamin K-dependent γ -carboxylation system. Indeed, subsequent research showed that calumenin was a potent inhibitor of the 94-kDa γ -carboxylase (Wajih *et al.*, 2004) and γ -carboxylase activity was significantly enhanced when calumenin was silenced by siRNA in BHK21 cells. In the same article, evidence is presented that calumenin is physically associated with γ -carboxylase in the ER membrane. The most important finding in these early studies was the identification of calumenin as the first known endogenous inhibitor of the vitamin K-dependent γ -carboxylation system. The inhibitor belongs to the ER-located CREC subfamily of Ca²⁺ binding proteins which all contain an EF-hand motif that binds Ca²⁺ (Honore and Vorum, 2000). All proteins have C-terminal retrieval sequences (HDEL, HDEF, and HEEF) for retention either in the ER or the Golgi apparatus (Honore and Vorum, 2000). Despite being a hydrophilic water-soluble acidic protein, calumenin is associated with ER membrane proteins. This finding comes from EM studies of the ER membrane (Vorum *et al.*, 1999) and our demonstration that calumenin is strongly associated with lipid-detergent micelles derived from the ER membrane which also carry the VKOR enzyme complex (Wallin *et al.* 2001).

A. Identification of protein disulfide isomerase to be involved in reduction of the CXXC redox center in VKORC1

The discovery of VKORC1 and calumenin being parts of the vitamin K-dependent γ -carboxylation system directed focus on the *in vivo* system responsible for reduction of the CXXC redox center in VKORC1. Since thioredoxin (Preusch, 1992) and glutathione (Whitlon *et al.*, 1978) had been eliminated as reductants of VKORC1, we still found the hypothesis interesting that the dominant chaperone and redox protein disulfide isomerase (PDI) involved in oxidative folding and isomerization of proteins in the ER was a candidate reductase for VKORC1. Our hypothesis was also inspired by earlier work published by Soute *et al.* (1992) which suggested that PDI could be involved, but their work did not provide conclusive conformation of PDI involvement. For a long time, it was not understood where the electrons resulting from thiol-dependent protein folding ended up. With the discovery of endoplasmic reticulum oxidase 1 (ERO1), it became clear that ERO1 oxidizes reduced PDI and produces H_2O_2 by reducing O_2 (Gross *et al.*, 2006). We proposed that part of this protein folding generated an “electron pool” that could be used to make the reduced γ -carboxylase vitamin K_1H_2 cofactor. Our success in finding that PDI indeed is involved in reduction of the CXXC center in VKORC1 was the use of extensively washed ER membranes where all the luminal and peripherally bound membrane proteins were removed before the membranes were used for assays and in search for proteins associated with the vitamin K-dependent γ -carboxylation system which is embedded in the lipid bilayer of the ER membrane (Wallin *et al.*, 2002). As shown in Fig. 11.6A, we (Wajih *et al.*, 2007) found that oxidative folding in the presence of vitamin K_1 2,3-epoxide and reduced RNase was almost as effective as chemically reduced vitamin K_1H_2 in triggering γ -carboxylase activity as cofactor. Importantly, we could also show that the PDI inhibitor bacitracin inhibited the reduced RNase triggered (Fig. 11.6B) but not the DTT-triggered reduction of the epoxide (Fig. 11.6C). Additionally, we could show that siRNA against PDI in HEK293 cells significantly reduced RNase-triggered VKOR activity (Fig. 11.6D1 and D2). These data strongly suggested that PDI, as an oxidative thiol refolding chaperone of reduced RNase in our test system, was involved in reduction of the CXXC center in VKORC1. The reduced RNase-triggered reduction of vitamin K 2,3-epoxide was inhibited by warfarin. In the ER, PDI is a known subunit of triglyceride transfer protein and prolyl hydroxylase (Koivunen *et al.*, 2005) and binding of proteins to PDI is known to involve PDI's hydrophobic b' domain (Zhao *et al.*, 2005). We proposed that a complex was formed between PDI and VKORC1 as part of the hypothetical redox exchange reactions going on between the reduced CGHC center in the a' or a domain (Wajih *et al.*, 2007) in PDI and the oxidized CXXC center in VKORC1.

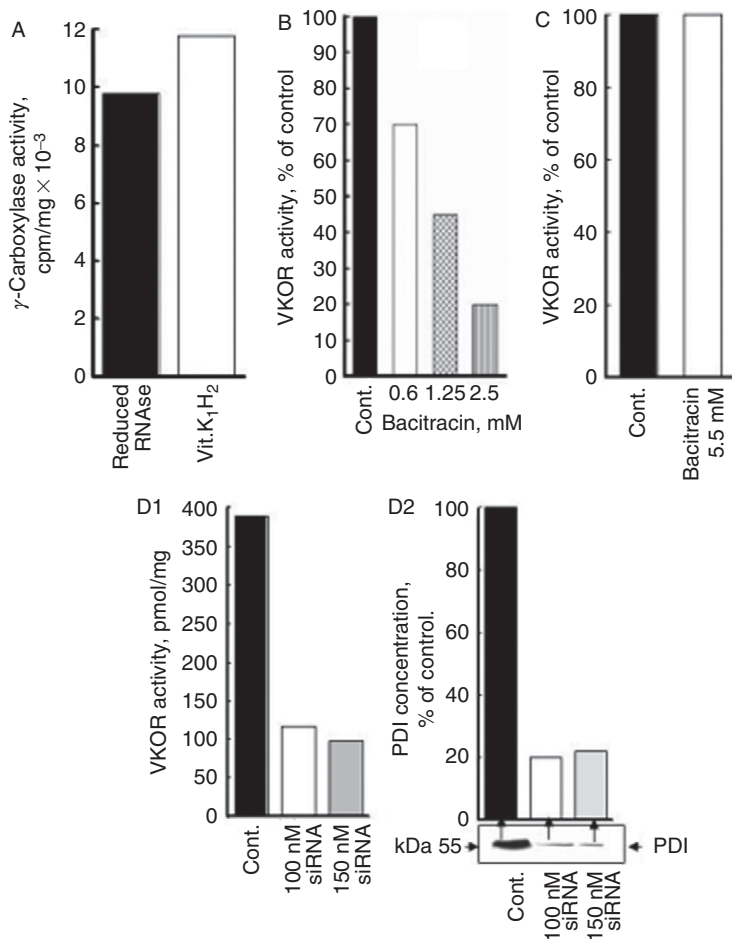


Figure 11.6 Oxidative thiol-dependent protein folding is involved in reduction of the CXXC redox center in VKORC1 which triggers reduction of vitamin K_1 2,3-epoxide (Vit. $K > O$) to the reduced vitamin K_1H_2 (Vit. K_1H_2) γ -carboxylase cofactor by VKORC1. Panel A shows that reduced RNase in the presence of Vit. $K > O$ can trigger γ -carboxylation of the synthetic γ -carboxylase peptide substrate FLEEL almost to the same extent as chemically reduced vitamin K_1H_2 . Panel B shows that reduced RNase can trigger VKOR activity and that the reduced RNase-triggered activity is inhibited by the PDI inhibitor, bacitracin. Panel C shows that DTT-triggered VKOR activity is not inhibited by bacitracin. Vitamin K_1H_2 -triggered FLEEL γ -carboxylation was also shown not to be inhibited by bacitracin (data not shown). Panel D1 shows that siRNA silencing of PDI in HEK293 cells inhibits VKOR activity. Panel D2 shows reduction of PDI concentrations in HEK293 cells treated with 100 and 150 nM siRNA against PDI.

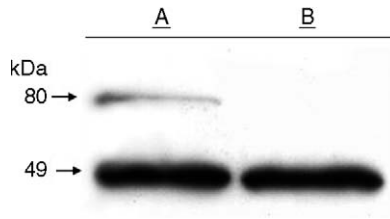


Figure 11.7 Identification of an 80-kDa PDI–VKORC1 complex in HEK293 cells stably transfected with a *c-myc*–VKORC1 cDNA construct. Cells were lysed and immunoprecipitated with goat anti-*c-myc* antibodies and preimmune goat IgG, respectively. The immunoprecipitates were Western blotted with monoclonal mouse PDI antibodies and lanes A and D show the results obtained when the blots were visualized with secondary goat anti-mouse PDI antibodies. The 49-kDa bands in both lanes were shown to result from cross-reaction of the secondary antibody with the heavy chain of goat IgG.

An extensive number of experiments including immunoprecipitation, 1D- and 2D-SDS-PAGE, Western blotting, and mass spectrometry (MS/MS) were used to search for the complex. These experiments are described in detail in [Wajih *et al.* \(2007\)](#) and provided evidence for the existence of an 80-kDa enzyme complex containing VKORC1 and PDI. We also acquired additional data supporting the existence of a PDI–VKORC1 complex in cells transfected with a *c-myc*-tagged VKORC1 construct. In this experiment, we isolated the complex with goat anti-*c-myc* antibodies. As shown in [Fig. 11.7](#), the monoclonal PDI antibodies recognized a protein band in lane A, which contains proteins from the immune complex isolated with the *c-myc* antibodies, but this protein band was absent in lane B, which contains proteins from the control incubation with preimmune goat IgG. A protein band of 49 kDa was seen at equal intensity in both lanes. The identity of this contaminating protein has been identified as the heavy chain of goat IgG recognized by the secondary antibody used to detect the monoclonal mouse anti-PDI antibody. From these experiments, a new and extended hypothetical *in vivo* version of the vitamin K-dependent posttranslational γ -carboxylation modification system has emerged. The system is depicted in [Fig. 11.8](#) and includes our current vision on how thiol oxidative protein folding is linked to γ -carboxylation of the vertebrate members of the vitamin K-dependent protein family including the systems association with the inhibitor, calumenin.

B. Discovery of VKORC1 and calumenin has improved synthesis of functional recombinant coagulation factors

Recombinant human factors VII, IX, and protein C have become valuable recombinant pharmaceuticals in the treatment of bleeding disorders and sepsis ([Hedner, 2006](#); [Pipe *et al.*, 2004](#); [Russell, 2006](#)). However, a major

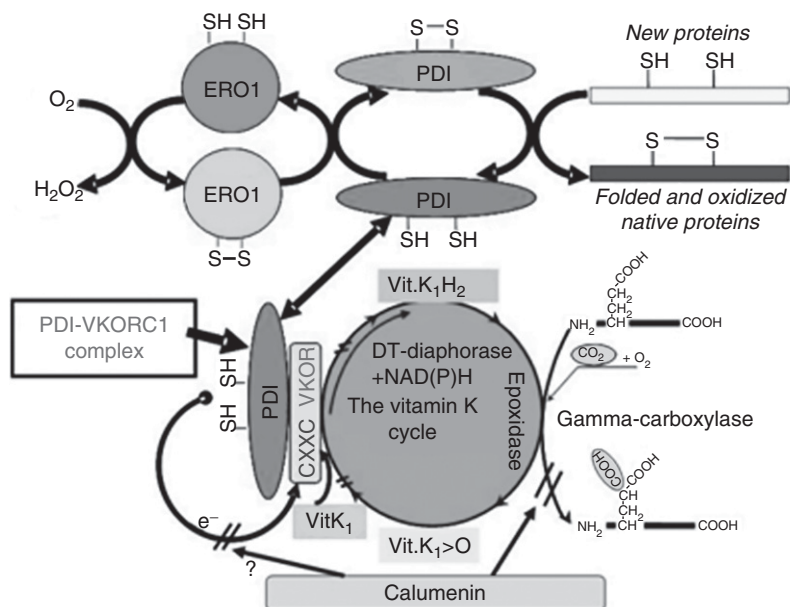


Figure 11.8 Dithiol-dependent oxidative protein folding in the ER is linked to post-translational γ -carboxylation of proteins, a hypothetical model. The thioredoxin-related redox centers CGHC (S-S) in PDI are reduced (SH-SH) when PDI catalyzes dithiol-dependent oxidative folding of newly synthesized proteins in the ER. Reoxidation of the redox centers by endoplasmic reticulum oxidase 1 (ERO1) is a major pathway for the continuous operation of PDI in oxidative folding. Our data suggest that some of the PDI proteins in the ER form stable complexes with the VKORC1 integral membrane protein. We propose that the PDI-VKORC1 protein complex is responsible for warfarin-sensitive reduction of Vit. K > O by the vitamin K cycle to form reduced vitamin K₁H₂, the essential cofactor for γ -carboxylation of vitamin K-dependent proteins. Calumenin is shown as an inhibitor of γ -carboxylase and potentially also the warfarin-sensitive VKOR, K₁ > O reducing enzyme complex.

problem with production of recombinant vitamin K-dependent coagulation factors for use as pharmaceuticals has been poor recovery of functional proteins from the cell medium. Following cloning of γ -carboxylase, it was shown, surprisingly, that overexpression of γ -carboxylase in cells stably overexpressing recombinant human factor IX (r-hFIX) (Rehementulla *et al.*, 1993) inhibited synthesis of functional r-hFIX. However, the discovery of the *VKORC1* gene has provided new opportunities to understand the system. When *VKORC1* is expressed in various cell lines, *VKORC1* increases reduced vitamin K cofactor production and overexpression of *VKORC1* increases the capacity of the cells' γ -carboxylation system (Wajih *et al.*, 2005a, b). *VKOR* has been shown by several laboratories to be the rate-limiting step in the system. This finding provides new opportunities for increased production of fully γ -carboxylated and functional

recombinant vitamin K-dependent proteins. We have produced a stably transfected cell line with an engineered γ -carboxylation system that produces 80% functional recombinant human factor IX (r-hFIX) compared to 18% production by the same cell lines engineered to only overexpress r-hFIX (Wajih *et al.*, 2005a). This dramatic increase (80%) in production yield of functional r-hFIX resulted from silencing of calumenin, the inhibitor of the vitamin K-dependent γ -carboxylation system in the ER (Wajih *et al.*, 2005a). In these initial studies, transient transfections with SMART POOL siRNA oligonucleotides against calumenin were used. Current engineering by our laboratory has produced cell lines which stably overexpress shRNA against calumenin in addition to VKORC1 and human factors IX, VII, and protein C. For the pharmaceutical industry, these cell lines may increase production yield of the functional clotting factors which should have an impact on medical cost and extent of treatment.

III. GENETICS OF WARFARIN RESISTANCE

Identification of the *VKORC1* gene by Oldenburg's group (Rost *et al.*, 2004) and their finding of mutations that were associated with warfarin resistance made a major breakthrough in a "long" search for the underlying mechanisms responsible for the resistance. Although the inter-individual variability in response to warfarin as an anticoagulant is multifactorial which include nutrition (Rohde *et al.*, 2007), drug metabolism by the Cyp2C9 gene (Rettie *et al.*, 2006), and potentially other factors in the ER (Wajih *et al.*, 2004), it has become more and more clear that the combined effect of altered VKORC1 and Cyp2C9 enzymes plays a significant role in defining the response that rodents and humans get by receiving the drug. Of the Cyp2C9 genes in humans, two common allelic variants, Cyp2C9*2 and Cyp2C9*3, encode P-450 enzymes that are only 12% and 12.5% as effective in hydroxylating and neutralizing the drug as the wild-type enzyme (Haining *et al.*, 1996; Rettie *et al.*, 1994; Sullivan-Klose *et al.*, 1996). However, the only knowledge on inactivation of warfarin by the P-450 system was not often enough to explain the often large variation in warfarin doses that were needed to anticoagulate patients with thromboembolic disease. Reider *et al.* (2005) have demonstrated that VKORC1 polymorphism can be grouped into two groups, A and B, where the mutations in the A group is associated with low dose warfarin requirement and the B group with high dose drug requirement. Recent studies have focused on genotyping Cyp2C9 and VKORC1 in patients and the outcome looks promising for the use of genotyping as a prescreening method that can be used to predict warfarin dose requirement by individuals. Also, warfarin has been and still is used extensively as a rodenticide in pest control. Because of

its extensive use as rodent poison, rodent strains resistant to warfarin have become a significant human health and agricultural problem. Before the *VKORC1* gene was identified, much toxicological research was carried out to deal with the warfarin-resistant problem and the second generation anticoagulants became one of the solutions. These compounds have extreme toxicity and rat strains resistant to the second generation anticoagulants have also been reported. Pelz *et al.* (2005) have mapped mutations in several warfarin-resistant strains of rats and this approach may be helpful in the design of new less toxic rodenticides to be used in pest control.

IV. FUTURE PERSPECTIVES

Discovery of the *VKORC1* gene will inspire to a variety of additional new experiments which will (1) expand our knowledge of *in vivo* posttranslational vitamin K modification of proteins, and (2) develop test systems used to genetically screen patients to provide information for safe and stable long-term use of warfarin to control thromboembolic disease. Further work on engineered cell lines used for production of recombinant vitamin K-dependent proteins used in medicine and research is highly likely to be performed. One remaining puzzle to be solved is to understand the mechanism by which the reduced vitamin K₁H₂ cofactor is “transported” from the PDI-VKORC1 complex to γ -carboxylase in the oxidative environment of the ER. Research is also expected to be focused on assembling a functional γ -carboxylation system *in vitro* from the purified components of the system which will include enzymes and regulatory proteins. Important components in a successful reconstitution process will be lipids. Current research work on the system is constantly reminding us on the essential part lipids play in functioning of the system but little is known about their functions at the molecular level.

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WARFARIN THERAPY: INFLUENCE OF PHARMACOGENETIC AND ENVIRONMENTAL FACTORS ON THE ANTICOAGULANT RESPONSE TO WARFARIN

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Abstract

Warfarin is difficult to use because of a marked inter- and intraindividual variability among patients in the required dosage. Recent advances in understanding the vitamin K cycle have been made. Besides well-known demographic or environmental factors (advanced age, vitamin K intake, concomitant drugs,

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comorbid conditions, and acute illnesses), genetic single nucleotide polymorphisms (SNPs) have been identified as strongly affecting the maintenance dosage and its variability. SNPs of vitamin K epoxide reductase complex subunit-1 (*VKORC1*) gene have been identified, affecting the enzyme shown as one of the target of vitamin K antagonist. SNPs of cytochrome P₄₅₀ 2C₉ (*CYP2C9*) gene have been shown to decrease the catabolism of warfarin. The combined analysis of *VKORC1*, *CYP2C9* SNPs, and age may account for more than 50% of the individual variability in the warfarin maintenance dosage. Predicting models of warfarin maintenance dosage taking into account these individual parameters are currently developed. © 2008 Elsevier Inc.

I. INTRODUCTION

In the twenties, two veterinarians, Schofield and Roderick, observed that cattle that ate spoiled sweet clover died of severe hemorrhages. In 1939, K. P. Link, an expert in the biochemistry of sweet clover, first isolated dicumarol from this plant. Continued work in Link's laboratory finally led to an anticoagulant more potent than dicumarol that, unlike dicumarol, was water soluble. To honor the Wisconsin Alumni Research Foundation that supported Link's research, the new compound was named warfarin. Finally, in 1947, C. Owen and colleagues showed that all the vitamin K-dependent factors were depressed by vitamin K antagonist (VKA).

Warfarin is currently the most widely used VKA worldwide. VKAs are prescribed to millions of people each year for the primary and secondary prevention of various arterial and venous thromboembolic diseases. However, VKAs are challenging to use in clinical practice ([Ansell *et al.*, 2004](#)). First, they have a narrow therapeutic index and laboratory control is needed. In the eighties, improvement in the safety and effectiveness of oral anticoagulant treatment resulted from the standardization of the prothrombin time test by the adoption of International Normalized Ratio (INR). Optimal therapeutic ranges with target INRs have been defined for each indication. However, major bleeding remains a feared adverse effect, especially during the first weeks of treatment. Second, VKAs are characterized by a marked inter- and inpatient variability. Since decades, it is well known that the maintenance dose of VKAs is influenced by different acquired factors, including demographic data, dietary vitamin K intake, comorbid conditions, acute illnesses, and comedications. About 10 years ago, recent advances in pharmacogenetics have been made and pharmacogenetic-based warfarin therapy could improve in the future the safety and effectiveness of this drug.

II. VITAMIN K AND VITAMIN K ANTAGONISTS AND BLOOD COAGULATION

Vitamin K was discovered in 1929 by the Danish Henrik [Dam \(1935\)](#) who mentioned that when newborn chicks were fed an extracted “cholesterol-free” diet, they often died from bleeding. Later on, he found out that a new fat-soluble substance, different from cholesterol, prevented or corrected the disease. This substance was called vitamin K for “Koagulation-Vitamin.” Vitamin K₁ was subsequently isolated from alfalfa and vitamin K₂ from fish meal. There are three classes of vitamin K ([Fig. 12.1](#)): phyloquinone or vitamin K₁ found mainly in green leafy vegetables such as kale, spinach, and broccoli; menaquinone or vitamin K₂ with an unsaturated isoprenyl side chain found in liver, milk, cheese, and fermented soy products ([Dam, 1935](#); [Stafford, 2005](#)); and menadione or vitamin K₃, which is a chemically synthesized derivative used for animal feed. The menaquinone is also synthesized by intestinal bacteria.

The main function of vitamin K is to act as a cosubstrate for carboxylation of the vitamin K-dependent proteins. This posttranslational modification of glutamate to γ -carboxyl glutamate is required for the activity of vitamin K-dependent proteins. Among the known vitamin K-dependent proteins are the procoagulant factors VII (proconvertin factor), IX (antihemophilic B factor), X (Stuart Factor), and II (prothrombin) and regulatory anticoagulant proteins (protein C, S, and Z). Once the vitamin K-dependent proteins have been γ -carboxylated, they can react with calcium, which is required for their binding to phospholipids and thus

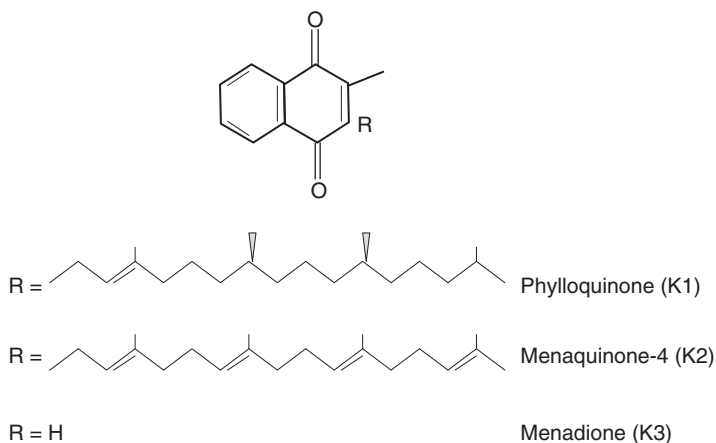


Figure 12.1 Different forms of vitamin K: phyloquinone (vitamin K₁); menaquinones (vitamin K₂); menaquinone-4; and menadione (vitamin K₃).

for their activity. Carboxylation is accomplished by the enzyme γ -glutamyl carboxylase which requires the propeptide-containing substrate and three cosubstrates: reduced vitamin K₁ (K₁H₂ or hydroquinone), CO₂, and O₂ (Stafford, 2005). Concomitant with γ -carboxylation, K₁H₂ is converted to vitamin K₁ epoxide. The epoxide is then reduced by the vitamin K₁ 2,3-epoxide reductase (VKOR) in a two-step reaction to vitamin K (quinone) and then to vitamin K₁H₂ (Wallin and Hutson, 2004). This vitamin K cycle is depicted in Fig 12.2.

Treatment with VKAs results in the hepatic production of partially carboxylated and decarboxylated proteins with reduced coagulant activity (protein induced by vitamin K antagonists: PIVKA). The anticoagulant effect of VKAs depends more on the half-life of procoagulant factors than on that of VKAs: the half-life of factor VII is shortest at 6 h, that of factor II is longest at 60 h, and that of factors IX and X are intermediate at 24 and 36 h.

When the liver concentration of vitamin K₁ is elevated, vitamin K₁ can be reduced by an alternative pathway in the liver and provide the

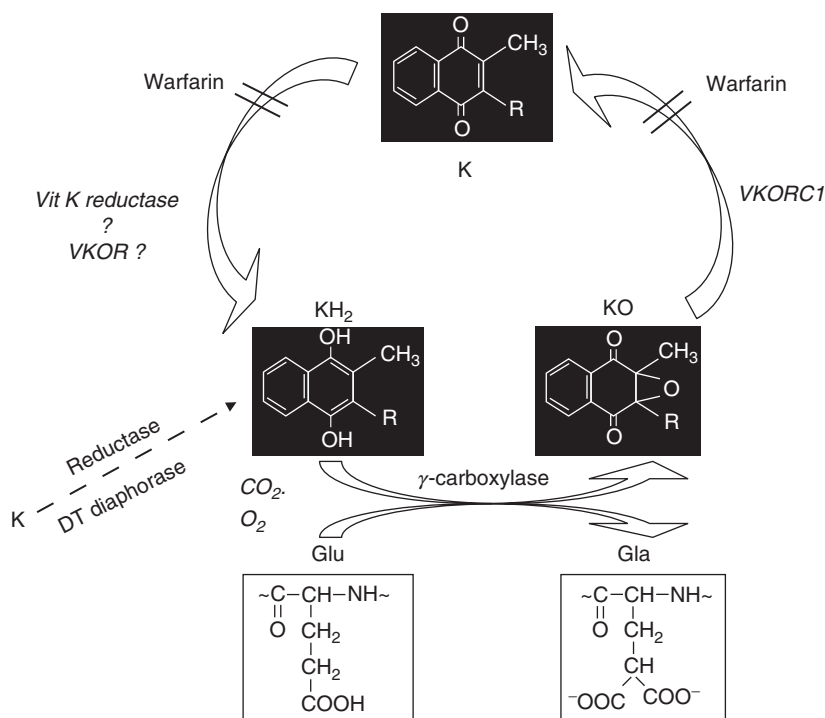


Figure 12.2 Vitamin K cycle. K: Vitamin K₁ quinone; KH₂: Vitamin K₁ hydroquinone; KO: Vitamin K₁ epoxide; VKORC1: Vitamin K oxidoreductase.

γ -glutamyl carboxylase with cofactor (Fig. 12.1). This pathway is catalyzed by DT-diaphorase. Warfarin does not inhibit these enzymes. Therefore, if the VKOR pathway for vitamin K₁ reduction is blocked by warfarin, the alternative can drive K₁H₂ cofactor production and maintain a normal functioning blood coagulation system. This is the standard procedure used to treat warfarin intoxication (Wallin and Hutson, 2004).

III. PHARMACOLOGY OF WARFARIN

A. VKOR: The target of warfarin

The gene that codes for epoxide reductase was recently cloned concomitantly by two independent teams, whereas the activity of the enzyme was first reported in 1970 (Li *et al.*, 2004; Rost *et al.*, 2004).

Coumarins are effective rodenticides, causing fatal bleeding in coumarin-naïve rodents. Studies of wild-caught, coumarin-resistant rats have provided important clues to the location of VKOR. The second clue to the location of VKOR came from a study of familial multiple coagulation factor deficiency, which is an extremely rare, autosomal recessive, bleeding disorder characterized by inadequate γ -carboxylation of coagulation factors II, VII, IX, and X (Gage, 2006). A first single protein component of the putative VKOR enzyme complex has been identified by genetic linkage analysis and positional cloning based on sequence similarity between rat and human (Rost *et al.*, 2004): to identify the gene, they directly sequenced genomic DNA in a 4-Mb region on chromosome 16 from two probands with familial multiple coagulation factor deficiency and four unrelated patients with warfarin resistance. They found a 5,126 base-pair gene of three exons coding a 163-amino acid protein with a mass of 18.2 kDa (Fig. 12.3). They named the gene vitamin K epoxide reductase complex subunit 1 (*VKORC1*). They confirmed *VKORC1* function by transfecting human embryonic kidney cells with the novel gene (Gage, 2006). Independently, in the same issue of *Nature*, Li and colleagues (2004) used positional cloning approach after mapping of warfarin resistance to rat chromosome 1 and of vitamin K-dependent protein deficiencies to the syntenic region of human chromosome 16 to identify *VKORC1* in the chromosome region 16p12-q21. They focused on 13 candidate genes and used short interfering RNA pools against individual genes to test their ability to inhibit VKOR activity in human cells (Li *et al.*, 2004). They confirmed the activity of *VKORC1* by transfecting insect cells with the novel gene and demonstrating that the cells gained warfarin-sensitive *VKORC1* activity (Gage, 2006). The 5 kb *VKORC1* gene encodes a protein of 163 amino acids with a mass of 18 kDa. VKOR is an integral membrane enzyme in the endoplasmic reticulum (ER) that exhibits broad

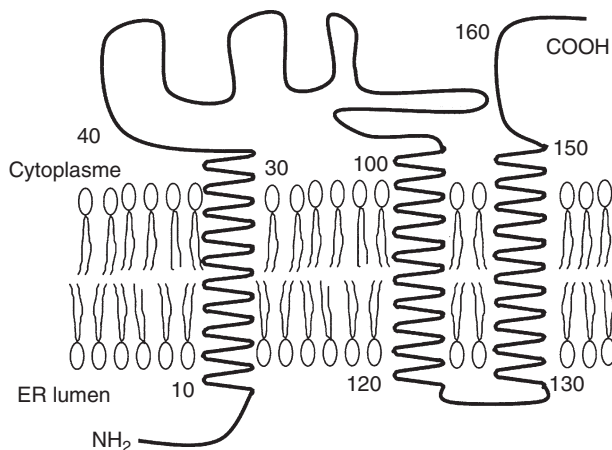


Figure 12.3 Structure of VKORC1 according to [Stafford \(2005\)](#). ER: endoplasmic reticulum.

tissue distribution ([Berkner, 2005](#)). Thiols are involved in the catalytic mechanism of vitamin K₁ epoxide reduction by VKOR ([Wallin and Hutson, 2004](#)). The most plausible model for the mechanism of inhibition of VKOR is that proposed by [Fasco *et al.* \(1983\)](#): warfarin binds to an oxidized thiol redox center in VKOR and prevents reduction of the center by an unknown electron donor ([Fasco *et al.*, 1983](#); [Wallin and Hutson, 2004](#)).

Of note, progress in genetics has been very useful in order to understand the mechanism of resistance to VKA in patients, which is very rare: few different mutations on *VKOR* gene have been associated with this resistance ([Bodin *et al.*, 2005](#)).

B. Pharmacokinetics of warfarin

Warfarin is a racemic mixture of equal amounts of (*R*)- and (*S*)-warfarin ([Ansell *et al.*, 2004](#); [Jacobs, 2006](#)). The (*S*)-enantiomer has 3 to 5 times greater anticoagulation effect than its optical congener. The bioavailability of both warfarin enantiomers after oral administration is almost complete. Both enantiomers are bound to plasma proteins (plasma unbound fractions of 1%) and are extensively eliminated by hepatic metabolism in humans. The half-life of warfarin is about 40 h. The cytochrome P450 (*CYP2C9*), one of the major isoforms of the *CYP2C* family (about 20% of constitutively expressed hepatic CYP in humans), is almost exclusively responsible for the metabolism of the (*S*)-enantiomer in humans ([Kirchheiner and Brockmöller, 2005](#)). The (*S*)-warfarin is extensively metabolized to its

major metabolite (*S*)-7-hydroxy-warfarin and to a much lesser extent to (*S*)-6-hydroxy-warfarin. Both metabolites are inactive and are excreted in the bile. The pharmacologically less active (*R*)-warfarin is also metabolized by multiple isoforms of CYP (CYP1A2, CYP2C19, and CYP3A4) to an inactive alcohol that is excreted in the urine. Finally, consistent data indicate that the influence of *R*-warfarin on the overall anticoagulation effect is insignificant. Hepatic CYP2C9 activity is one of the prime determinants of the variability on the warfarin dosage (Jacobs, 2006).

C. Vitamin K and warfarin reversal

If warfarin reversal is required, the method chosen should reflect the clinical seriousness of bleeding and balance against the thrombotic risk of a temporary suspension/reduction of anticoagulation. Factors that require considerations include the indication for warfarin treatment, the seriousness of bleeding, and the speed and completeness of reversal required (Hanley, 2004). For warfarin reversal, options include simple omission ultraslow reversal, over days, administration of oral or intravenous (reversal: within 24 h) vitamin K and prothrombinic complex concentrate (rapid reversal within 10–15 min) (Hanley, 2004). In this setting, recommendations from the Seventh American College of Chest Physicians Conference on Antithrombotic and Thrombolytic Therapy have been established (Ansell *et al.*, 2004). Higher doses of vitamin K than those recommended should be avoided, as they may lead to overcorrection and resistance to warfarin for several days.

Vitamin K₁ is available as oral tablets or as ampoules for intravenous or oral administration. The subcutaneous or intramuscular administrations are not recommended. Indeed, the oral vitamin K has been shown to lower INR more rapidly than subcutaneous route (Crowther *et al.*, 2002). The intramuscular administration in patients on anticoagulation therapy (particularly when overanticoagulated) is associated with a significant risk of hematoma.

IV. INFLUENCE OF ENVIRONMENTAL FACTORS ON THE ANTICOAGULANT RESPONSE TO WARFARIN

A. Diet and vitamin K status

Human requirements for vitamin K are obtained both by endogenous production by intestinal bacteria in the form of menaquinones and dietary intake of vitamin K₁. The Recommended Dietary Allowance (RDA) for vitamin K₁ is about 1 µg/kg of body weight/day (Booth *et al.*, 1997). Among commonly consumed foods, vegetables provide the highest amount of vitamin K₁, with the dark green vegetables, particularly leaves, being the richest sources. Per 100 g of food, the amount of vitamin K is 324 µg for

spinach, 88 μg for broccoli, 73 μg for cabbage, <40 μg for green pees, celery, and carrots, and <5 μg for tomatoes and root vegetables. Soybean oil, olive oil, and margarine contain 27, 7, and 5 μg of vitamin K per 100 g, respectively (Booth *et al.*, 1997). As vitamin K is a fat-soluble vitamin, the presence of fat in the meal is critical for absorption (Booth *et al.*, 1997).

It is only recently that studies have assessed the effect of dietary changes or inconsistencies on anticoagulant stability. Thirty-nine patients on warfarin, with two consecutive INR values within the therapeutic range at least 1 week apart, participated in a randomized crossover diet protocol after assessment of their usual vitamin K intake (Franco *et al.*, 2004). The mean baseline vitamin K intake was $118 \pm 51 \mu\text{g}/\text{day}$. They were randomly assigned to a diet enriched in (500% increase) or depleted of (80% decrease) vitamin K for 4 consecutive days, with 1- to 2-week washout period between diets (Franco *et al.*, 2004). The INR increased progressively among patients on vitamin K-depleted diet, from 2.6 ± 0.5 at baseline to 3.3 ± 0.9 on day 7 ($p = 0.05$), while after the enriched diet, the INR decreased from 3.1 ± 0.8 at baseline to 2.8 ± 0.6 on day 4 ($p = 0.04$). In a series of anticoagulated patients consuming their usual diets, vitamin K intake >250 $\mu\text{g}/\text{day}$ decreased sensitivity to warfarin (Lubetsky *et al.*, 1999). The threshold dose of vitamin K₁ supplements causing a significant decrease in the INR was identified at 150 $\mu\text{g}/\text{day}$ in healthy subjects with an INR change clinically relevant in 25% of the participants (Schurgers *et al.*, 2004). In 53 patients on warfarin therapy who had stably controlled anticoagulation, each increase of 100 μg in the daily dietary intake of vitamin K averaged over 4 days caused a reduction of only 0.2 in the INR value. The results of this study also showed that dietary enrichment with K had no effect on warfarin dose requirement while both age and CYP2C9 genotype influenced warfarin dose requirement (Khan *et al.*, 2004).

Even though several studies have shown that vitamin K intake plays an independent role in INR fluctuations in patients taking anticoagulants (Couris *et al.*, 2006; Cushman *et al.*, 2001; Custodio das Dores *et al.*, 2007; Franco *et al.*, 2004; Khan *et al.*, 2004; Lubetsky *et al.*, 1999; Pedersen *et al.*, 1991), the magnitude of INR variations is low and of low clinical relevance (Franco *et al.*, 2004; Khan *et al.*, 2004), and the amount of vitamin K intake that influence INR is much higher than the RDA (Couris *et al.*, 2006; Franco *et al.*, 2004).

However, many physicians advise their patients on warfarin to restrict their consumption of vitamin K₁-rich food. Such a restriction may lead to instability of the anticoagulation since a constant level of dietary vitamin K intake is important in achieving stable anticoagulation (Franco *et al.*, 2004; Reese *et al.*, 2005; Sconce *et al.*, 2007). Moreover, the approach of restricting vitamin K intake to improve stability of anticoagulant control is not appropriate to maintaining optimal function of vitamin K-dependant

proteins and could lead to deficiencies of other nutrients such as carotenoids found in the same food (Khan *et al.*, 2004). In a study of 40 postoperative patients, at the onset of warfarin therapy, patients with lower vitamin K status were more sensitive to warfarin than patients with higher vitamin K status (Cushman *et al.*, 2001) and thus more prone to overanticoagulation. Sorano *et al.* (1993) reported that in patients with poor coagulation control, the stability of oral anticoagulant therapy is improved with controlled vitamin K₁ content. More recently, Sconce *et al.* (2005a) demonstrated that patients with unstable control of oral anticoagulation had a mean daily intake of vitamin K significantly lower than that for stable subject. Thus, to achieve stable anticoagulation in patients receiving chronic oral anticoagulation, dietary intake of vitamin K should be actively evaluated and taken into consideration. It is possible that much of the instability of anticoagulant control in patients on chronic warfarin therapy is attributable to a low and variable intake of dietary vitamin K and that stabilization of vitamin K intake in such patients by daily supplementation with oral vitamin K could result in more stable control of anticoagulation (Khan *et al.*, 2004; Sconce *et al.*, 2007). The precise amount of dietary vitamin K to be offered is not well established or completely evidence based (Rohde *et al.*, 2007). Supplementation with daily low-dose oral vitamin K (100 µg) significantly increased the number of INRs in range as well as the time spent in range, in a small series of selected patients treated with warfarin (Reese *et al.*, 2005). In a recent double-blind, randomized, placebo-controlled trial, Sconce *et al.* (2007) demonstrated that 150 µg of vitamin K along with warfarin therapy can lead to a more stable anticoagulation in patients with atrial fibrillation.

A rising number of adults consume multivitamin supplements daily. Most over-the-counter multivitamin supplements contain small amounts (10–25 µg) of vitamin K₁ and thus should not influence the stability of anticoagulation in patients on oral anticoagulants. However, the results of one study suggest that in anticoagulated patients with very low vitamin K₁-plasma status, over-the-counter multivitamin supplements significantly reduce warfarin effect (Kurnik *et al.*, 2004). As mentioned earlier, this study emphasizes the notion that patients with very low vitamin K₁-plasma status are sensitive to even small changes in vitamin K₁ intake and more prone to unstable anticoagulation.

B. Interfering drugs with warfarin

Drug interactions are frequent causes of adverse events with oral anticoagulant. Recently, Holbrook *et al.* (2005) provided an updated systematic overview of the quality, clinical effect, and importance of these reported interactions. They showed that there is an abundance of medications for which an adverse interaction with warfarin, generally potentiation of warfarin's

effect, has been reported. Among these drugs are those commonly used such as antibiotics, azoles (fluconazole, miconazole, and metronidazole), amiodarone, selective serotonin reuptake inhibitors, omeprazole, and lipid-lowering drugs (Holbrook *et al.*, 2005, Panneerselvam *et al.*, 1998).

Although the true mechanisms of drug interactions almost remain unknown, there are several pharmacokinetic and pharmacodynamic factors that could influence warfarin's effect. Many of the drugs potentiating warfarin's effect are known as inhibitors of CYP2C9, including amiodarone, fluconazole, fluvastatin, lovastatin, fluvoxamine, isoniazid, and phenylbutazone (Holbrook *et al.*, 2005). The pharmacodynamics of warfarin may be influenced by medications that interfere with the endogenous synthesis of vitamin K: for instance, antibiotics eliminate bacterial flora and aggravate vitamin K deficiency. Of note, acetaminophen, which is the first-line analgesic and antipyretic therapy in patients receiving oral anticoagulants, was shown to induce, only at doses greater than 4 g/day, a significant increase in INR in patients receiving a stable regimen of warfarin (Mahé *et al.*, 2005). In contrast, the anticoagulant effect of warfarin may be inhibited by enzymatic inducers like rifampicin, carbamazepine, and barbiturates or by drugs that reduce warfarin's gastrointestinal absorption such as cholestyramine (Holbrook *et al.*, 2005). Holbrook *et al.* (2005) conclude in their review that understanding a drug's pharmacology helps predict its potential for interaction with warfarin but the translation of these predictions into clinical reality is far from certain. Thus, when changes in concomitant drugs occur, and particularly when drugs known to potentiate the effect of vitamin K antagonists are added, oral anticoagulation should be closely monitored to detect excess anticoagulation early and, thereby, prevent potential bleeding complications.

The most difficult drugs to deal with are those that potentiate bleeding on their own: other anticoagulants (heparin), antiplatelets drugs, and all nonsteroidal anti-inflammatory drugs (NSAIDs) including COX-2 selective NSAIDs. The risk of bleeding is then greater when taken with oral anticoagulants, and INR monitoring is of no help (Pautas *et al.*, 2006). These drugs should be managed with caution in combination with oral anticoagulants.

Widespread use of herbal medicines among patients on warfarin is of concern, as these remedies can potentiate its anticoagulant effects. Moreover, few of these patients report the use of herbal medicines to their physician. Some herbs have been found to alter the pharmacokinetics of warfarin. The Chinese herb Danshen, commonly used in China for the treatment of cardiovascular and cerebrovascular disease, has been found to significantly increase the absorption and decrease the clearance of warfarin. Asian ginseng was found to influence the clearance of warfarin (Jiang *et al.*, 2006). *Ginkgo biloba* inhibits CYP450. Some herbal remedies have an intrinsic anticoagulant (geum japonicum) or antiplatelet (garlic, ginger, and clove) properties (Samuels, 2005), and perioperative bleedings have been reported

in patients taking herbal medicines such as garlic, ginkgo, and ginseng (Ang-Lee *et al.*, 2001). In contrast, St. John's wort influences the pharmacokinetics of warfarin by CYP2C9 induction (Jiang *et al.*, 2006).

C. Age and comorbid conditions

Advanced age is associated with a low warfarin maintenance dose, independently of the presence of comorbid conditions or comedications. The decrease in the maintenance dose has been estimated at about 10% by decade (Gage *et al.*, 2004). Indeed, the mean warfarin maintenance dose is around 6 mg in 30-year-old patients versus around 4 mg in 70-year-old patients and ~3 mg in octogenarians (Redwood *et al.*, 1991; Pautas *et al.*, 2006). This increased sensitivity of older patients to VKAs is poorly understood: pharmacokinetic changes seem to be negligible and pharmacodynamic changes have not been well characterized (Hylek, 2001). It has been demonstrated that patients with low body weight and patients with stable comorbid conditions such as congestive heart failure, liver disease, and severe renal failure require lower doses (Siguret *et al.*, 2005). Furthermore, acute illnesses such as fever or diarrhea may be accompanied by a decrease in the maintenance dose (Penning-van Beest *et al.*, 2001).



V. INFLUENCE OF PHARMACOGENETICS ON THE ANTICOAGULANT RESPONSE TO WARFARIN

A. SNPs of CYP2C9 and interpatient variability

A decade ago, the influence of pharmacogenetics of CYP2C9 on the elimination of warfarin was elucidated. Human *CYP2C9* gene, which is located on chromosome 10, spans 55 kb: it contains nine exons that encode a 489-amino acid protein (Kirchheiner and Brockmöller, 2005). Single nucleotide polymorphisms (SNPs) leading to single amino acid changes have been identified. Beside CYP2C9*1 (wild-type allele) (Arg144/Ile359), allelic variants have been designated as CYP2C9*2 (Cys144/Ile359) and CYP2C9*3 (Arg144/Leu359). The allelic frequency is quite different across the ethnic groups. The CYP2C9*2 and CYP2C9*3 alleles were detected in 8–19% and 6–10% of Caucasians, in 0% and 1.7–5% of Asians (Chinese and Japanese), and in 1–3.6% and 0.5–1.5% of African-Americans, respectively (Ansell *et al.*, 2004; Takahashi and Echizen, 2001). Among Caucasians, nearly 30% of the subjects carry at least one mutated allele. Other SNPs, such as CYP2C9*4 (Arg144/Thr359) identified in Japanese or CYP2C9*5 (Asp360Glu) identified in African-Americans, are registered at the Web site of the Human CYP Allele Nomenclature Committee; their frequency is much rarer.

Several studies demonstrated that the *in vitro* catalytic activities of CYP2C9*2 and CYP2C9*3 enzymes for (S)-warfarin hydroxylation were strongly decreased as compared with that of CYP2C9*1 in various expression systems: CYP2C9*2 mutation reduced affinity for P450 oxidoreductase, whereas CYP2C9*3 reduced affinity for substrate (Takahashi and Echizen, 2001). The effects of different CYP2C9 genotypes on the *in vivo* elimination of warfarin were then investigated. The decrease of the S-warfarin clearance caused by CYP2C9 variants produce changes in anticoagulation response (Linder *et al.*, 2002). Both CYP2C9*2 and CYP2C9*3 variant alleles have been associated with decreased warfarin dose requirements (Table 12.1). The average warfarin maintenance dosage required in patients with CYP2C9*2/*1 is reduced by 13–22% compared with the corresponding dosage from patients with CYP2C9*1/*1 (wild type) with a target INR of 2.5; in patients with CYP2C9*3/*1, it is reduced by 21–49% compared with the corresponding dosage from patients with CYP2C9*1/*1. Warfarin maintenance dosage is much more reduced in CYP2C9*2/*2 or CYP2C9*3/*3 homozygotes or in compound heterozygotes (CYP2C9*2/*3). Of note, few data are available regarding the warfarin dose required by CYP2C9*3/*3 homozygotes: these patients have such a low warfarin dose requirement that stabilization is not possible and treatment with warfarin is often abandoned. Indeed, numerous studies confirmed a significant association between CYP2C9 genotype and warfarin sensitivity. For instance, in 561 warfarin-anticoagulated patients with a target INR of 2.5, Taube *et al.* (2000) showed that the maintenance dose of warfarin was significantly related to genotype ($p = 0.001$). Even in very elderly

Table 12.1 Daily warfarin maintenance dose in Caucasian patients (2.5 target INR)

CYP2C9	n	*1/*1	*1/*2	*2/*2	*1/*3	*2/*3	*3/*3
Margaglione <i>et al.</i> , 2000	180	6.7	5.2	ND	3.8	1.8	ND
Taube <i>et al.</i> , 2000	561	5.0	4.3	3.0	3.9	4.1	ND
Loebstein <i>et al.</i> , 2001	156	6.5	5.2	ND	3.3	3.3	ND
Scordo <i>et al.</i> , 2002	93	5.6	3.9	2.9	2.9	2.6	1.2
Higashi <i>et al.</i> , 2002	185	5.6	4.9	4.1	3.3	2.0	1.1
Kamali <i>et al.</i> , 2004	121	4.1	3.6	ND	2.7	ND	ND
Siguret <i>et al.</i> , 2004*	126	3.1	2.7	1.2	2.9	2.3	ND

* Elderly patients.

INR, International Normalized Ratios; ND, Not Determined.

patients, which require quite low dose of warfarin, the number of CYP2C9*1 and CYP2C9*2 alleles is significantly associated with the maintenance dose (Siguret *et al.*, 2004). Other clinical implications such as the influence of CYP2C9 variant allele possession on bleeding complications, on the time to achieve the maintenance dose, and on overanticoagulation were also investigated (Aithal *et al.*, 1999; Higashi *et al.*, 2002; Margaglione *et al.*, 2000; Peyvandi *et al.*, 2004). In a 13-month retrospective cohort study conducted in 185 patients, Higashi *et al.* (2002) suggested that the CYP2C9*2 and CYP2C9*3 alleles are associated with an increased risk of overanticoagulation and of bleeding events among patients in a warfarin anticoagulation clinic setting. Compared with patients with the wild-type genotype, patients with at least one variant allele had an increased risk of above-range INRs [hazard ratio (HR) 1.40 (95% CI 1.03–1.90)]; the variant group required more time to achieve stable dosing [HR 0.65 (95% CI 0.45–0.94)], with a median difference of 95 days ($p = 0.004$); patients with a variant genotype had a significantly increased risk of a serious or life-threatening bleeding event [HR 2.39 (95% CI 1.18–4.86)] (Higashi *et al.*, 2002). Other studies confirmed that CYP2C9 SNPs are associated with a significant increased risk of bleeding during warfarin induction (Margaglione *et al.*, 2000; Voora *et al.*, 2005). Similar results have been obtained concerning other coumarin derivatives (acenocoumarol, phenprocoumon) (Visser *et al.*, 2004).

B. SNPs of VKORC1 and interpatient variability

It remains to be clearly understood why patients with the same CYP2C9 genotype have such a great standard deviation to the mean warfarin maintenance dose. This was partly elucidated with the discovery of the warfarin target gene, which encodes VKORC1. In a cohort of 147 warfarin-anticoagulated patients, D'Andrea *et al.* (2005) was the first to identify two common SNPs, 1173C>T in the intron 1 and 3730G>A in the 3' untranslated region. Regardless of the presence of confounding variables, the mean adjusted dose required of warfarin was higher among patients with VKORC1 1173CC genotype (6.2 mg) than among patients carrying the 1173CT (4.8 mg; $p = 0.002$) or the 1173TT (3.5 mg; $p < 0.001$). Rieder *et al.* (2005) conducted a retrospective study of 186 European-American patients receiving long-term warfarin maintenance therapy, described in a previous study (Higashi *et al.*, 2002). Ten common noncoding VKORC1 SNPs were identified, one of these SNPs being identical as that described by D'Andrea (2005): seven SNPs were significantly associated with the warfarin maintenance dose ($p < 0.001$). Of these seven highly significant SNPs, five were strongly correlated with one another (linkage disequilibrium $r^2 \geq 0.9$). A high-dose haplotype group (B) and a low-dose haplotype group (A) were identified: the mean maintenance dose of warfarin differed significantly among the three haplotype group combinations at

6.2 ± 0.3 mg/day for B/B, 4.9 ± 0.2 mg for A/B, and 2.7 ± 0.2 mg for A/A. *VKORC1* haplotype groups A and B explained $\sim 25\%$ of the variance dose. The molecular mechanism of this warfarin dose response appears to be regulated at the transcriptional level: [Rieder *et al.* \(2005\)](#) found that *VKORC1* mRNA levels varied according to the haplotype combination.

The prevalence of *VKORC1* genotypes differs by ethnic groups ([Rieder *et al.*, 2005](#)). Asian-Americans had a higher proportion of group A haplotypes, explaining the lower warfarin dose requirement in these patients compared to Caucasians. In contrast, African-Americans had a higher proportion of group B haplotypes, explaining the higher warfarin dose in these patients compared to Caucasians.

Overall, the detection of both *VKORC1* and *CYP2C9* genetic variants contributes to explain an important part of the interindividual variability of the warfarin dosage requirement: they accounted for 35% of the variance in warfarin dose in the cohort of 186 patients ([Rieder *et al.*, 2005](#)). In other cohorts of patients, the genetic variants accounted from 27% to 39% of the variance in warfarin dose ([Fig. 12.4](#)) ([D'Andrea *et al.*, 2005](#); [Rieder *et al.*, 2005](#); [Sconce *et al.*, 2005b](#); [Veenstra *et al.*, 2005](#)).

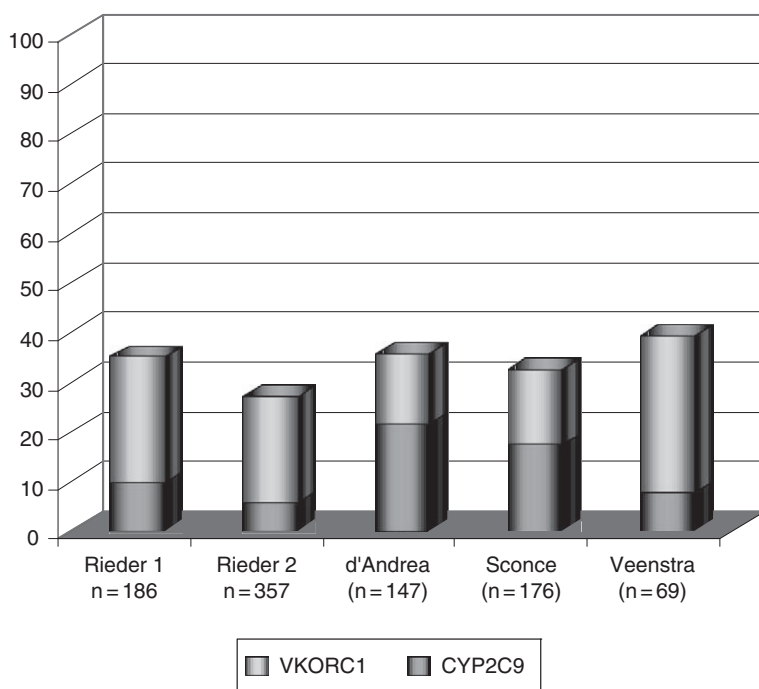


Figure 12.4 Effect of *VKORC1* and *CYP2C9* SNPs combination on the response to warfarin (percent in the variance warfarin). [Rieder *et al.* \(2005\)](#), [D'Andrea *et al.* \(2005\)](#), [Sconce *et al.* \(2005b\)](#), and [Veenstra *et al.* \(2005\)](#); *n* = number of patients.

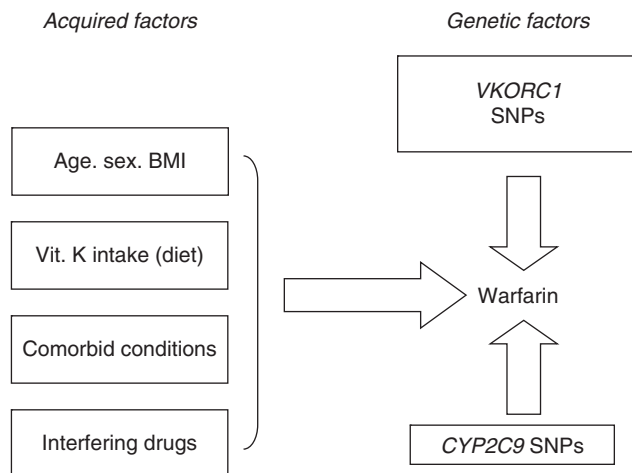


Figure 12.5 Demographic, environmental, and genetic factors affecting the warfarin maintenance dose.

VI. CONCLUSION

Among demographic and environmental factors affecting the warfarin maintenance dose, age and interfering drugs with warfarin appear as major determinants while vitamin K intake is a minor one. Besides, pharmacogenetic determinants have been identified as major determinants of warfarin sensitivity (Fig 12.5). Regression models have been derived from the combination of *VKORC1* and *CYP2C9* SNPs with clinical factors (weight, age, interacting drugs, and target INR), and dosing algorithms are currently proposed (Sconce *et al.*, 2005b; Voora *et al.*, 2005). This approach could reduce medical expenditure. Clinical studies are needed in this setting.

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VITAMIN K AND THROMBOSIS

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Abstract

Vitamin K was discovered in the 1930s during cholesterol metabolism experiments in chickens. It is a fat-soluble vitamin which occurs naturally in plants as phyloquinone (vitamin K₁) and is produced by gram-negative bacteria in the human gastrointestinal tract as menaquinone (vitamin K₂). This vitamin was found to be essential for normal functioning of hemostasis. In addition, a number of clinical conditions in which vitamin K deficiency was found to be the underlying pathophysiologic problem were discovered. These conditions include hemorrhagic disease of the newborn, obstructive jaundice, and malabsorption syndromes. The importance of this vitamin has become more apparent with the discovery of the anticoagulant warfarin which is a vitamin K antagonist. There are millions of patients on this therapy for a variety of thrombogenic conditions such as atrial fibrillation, deep vein thrombosis, pulmonary embolism, and prosthetic cardiac valves. The wide use of this narrow therapeutic index drug has resulted in significant risk for major bleeding. Vitamin K serves as one of the major reversing agent for patients over-anticoagulated with warfarin. In the past few years, research has focused on new areas of vitamin K metabolism, which include bone and endovascular metabolism; cell growth, regulation, migration, and proliferation; cell survival, apoptosis, phagocytosis, and adhesion. These new areas of research highlight the significance of vitamin K

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but raise new clinical questions for patients who must be maintained on long-term warfarin therapy. © 2008 Elsevier Inc.

I. INTRODUCTION

Vitamin K is a fat-soluble vitamin discovered in the 1930s when during cholesterol experiments in chickens it was observed that the chicks developed subcutaneous and intramuscular hemorrhages (Dam and Schonheyder, 1934). Further research identified vitamin K as the coenzyme for the carboxylase that converts glutamic acid to the corresponding γ -carboxyl residues critical to calcium binding of the clotting factors II, VII, IX, X, and proteins C and S which are integral to regulating hemostasis. Initially, vitamin K became the therapeutic intervention for clinical conditions associated with the deficiency of this vitamin (Table 13.1) (Martinez and Barsigian, 1998). With the introduction of the vitamin K antagonist warfarin in the 1950s and the expanded use of this agent in the past 20 years, the use of vitamin K has expanded to a new therapeutic area. More recently, scientific investigation has identified bone metabolism and arteriovascular health as other targets for vitamin K metabolism. This chapter will review vitamin K's pharmacology, therapeutic uses, and future research.

II. PHARMACOLOGY OF VITAMIN K

The dietary trace element vitamin K was discovered in the early 1930s by the Danish biochemist Henrik Dam. While performing cholesterol metabolism research in chickens, Dam observed that chicks reared on a

Table 13.1 Clinical conditions associated with vitamin K deficiency

- | |
|---|
| <ol style="list-style-type: none">1. Hemorrhagic disease of newborns2. Dietary insufficiency combined with administration of broad spectrum antibiotics3. Total parenteral nutrition4. Malabsorption of lipid-soluble vitamins because of obstructive jaundice (intrahepatic or extrahepatic)5. Malabsorption because of intrinsic diseases of the bowel:<ul style="list-style-type: none">Celiac diseaseSprueInflammatory bowel diseaseShort bowel syndrome |
|---|

Martinez and Barsigian, 1998.

diet free of sterols and poor in fat developed large subcutaneous and intramuscular hemorrhages (Dam and Schonheyder, 1934). He named the essential factor Koagulation (abbreviated vitamin K) because of its requirement for normal hemostasis (Dam, 1935). Later that decade, vitamin K₁ was isolated as a yellow oil from alfalfa and vitamin K₂ was found as a crystalline product from putrefied fish meal (Almquist, 1936; Binkley *et al.*, 1939; Mckee *et al.*, 1939). The first clinical trials with the administration of vitamin K occurred shortly thereafter on patients with obstructive jaundice or biliary problems since it was believed that the bleeding problems observed in these patients were a result of vitamin K deficiency. In addition, vitamin K was administered to newborns suffering from hemorrhagic disease or with a prolonged international normalization ratio (INR). This latter experiment was so effective that the mortality among newborn infants decreased from 4.6% to 1.8% (Zetterstorm *et al.*, 2006). Dam and Doisy received the Nobel Prize in 1943 for the discovery of vitamin K and its chemical structure.

The term vitamin K refers to a group of fat-soluble vitamins required for normal functioning of the hemostatic mechanism. Dietary vitamin K (Fig. 13.2) occurs naturally in two major forms: phyloquinone (vitamin K₁) and menaquinone (vitamin K₂) (Martinez and Barsigian, 1998; Vermeer and Hamulyak, 1991). Phyloquinone is found exclusively in plants, while menaquinone is produced by a series of congeners synthesized by gram-positive bacteria in the human gastrointestinal tract (Bjornsson, 1984). The vitamin K

Table 13.2 Vitamin K content of selected foods

Food	Vitamin K content ($\mu\text{g}/100\text{ g}$)
Bacon	46
Beef liver	93
Broccoli	175
Butter	30
Cabbage	125
Cheese	35
Chicken liver	7
Coffee	38
Eggs	11
Lettuce	129
Milk	1
Pork liver	25
Soybean oil	193
Spinach	415
Tea, Green	712

Martinez and Barsigian, 1998.

content in vegetables, meat, and dairy products is listed in Table 13.2 (Martinez and Barsigian, 1998). These congeners differ from one another in the length of the isoprenyl units that are substituted at the quinone ring. The natural vitamin K found in fish meal has seven isoprenyl units and is known as menaquinone-7. Menadione (vitamin K₃ or menaquinone 0) lacks an isoprenyl substitute at this position and is therefore a provitamin, which is converted to an active vitamin K (menaquinone-4) in the microsomes of the liver (Vermeer and Hamulyak, 1991).

Vitamin K is absorbed in both the small bowel and colon, with phytonadione absorbed primarily in the proximal small bowel and the menaquinone and menadione in the distal small bowel and colon, respectively. Phytonadiones and menaquinones' absorption occurs predominantly via the lymphatics. Both forms require bile salts and pancreatic juice for maximal absorption, which ranges from 40% to 80% depending on the source (Bloomstrand and Forsgren, 1968). Menadione, which is water soluble and enters the blood, is well absorbed even without bile salts. When oral doses of vitamin K₁ are given, the peak concentration occurs in ~2 h, with a decrease in the fasting level from 1 to 3 ng/mL occurring over 2–3 days (Shearer *et al.*, 1970; Wiss and Gloor, 1966). Approximately 30–50% of the administered dose is recovered in the feces over a 5-day period, with smaller amounts of between 8% and 30% appearing in the urine as polar metabolites. Because it is fat soluble, the absorption of vitamin K is significantly retarded by coadministration of nonabsorbable lipids, such as mineral oil. Vitamin K₁ (phyloquinone) administered parenterally will appear in the liver within 1 h (Bell and Matschiner, 1969). Within the hepatocytes, the largest concentration of vitamin K appears in the microsomal fraction, with the second largest in the cytosol. Other major uptake sites of this vitamin include the skin and muscle, and concentrations in these tissues reach a maximum ~24 h after parenteral administration (Bell and Matschiner, 1969). The body pool of vitamin K is not large and its turnover is rapid (Bjornsson *et al.*, 1979, 1980). Distribution in the body involves two phases. The initial disappearance from the plasma occurs with a half-life of ~25 min, and the longer, second phase with a half-life of ~160 min. The fractional turnover rate is 0.4 per h, thus indicating that the body pool turns over approximately once every 2.5 h (Bjornsson *et al.*, 1979). The minimal daily requirement is estimated between 0.03 and 1.50 $\mu\text{g/kg/day}$, with a portion being contributed through biosynthesis of menaquinone forms of vitamin K by the intestinal flora (Frick *et al.*, 1967). Because the body stores of vitamin K are small, partial depletion without clinical consequences can occur after 1 week of reduced dietary intake (Bjornsson *et al.*, 1980). Vitamin K deficiency will occur when the oral intake is decreased and bowel sterilizing antibiotics are used, thus eliminating the source of vitamin K (Frick *et al.*, 1967).

Vitamin K participates in the reaction that leads to the formation of the γ -carboxyglutamyl (Gla) residues required for the formation of active

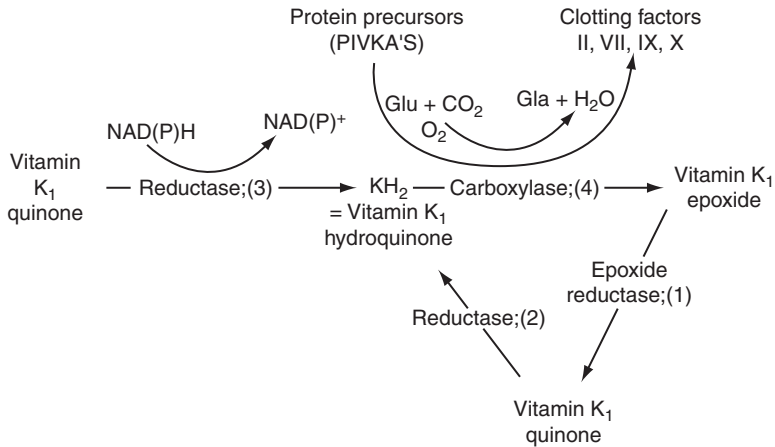


Figure 13.1 The vitamin K cycle. (1) and (2) are dithiol-dependent reductase enzymes that are inhibited by warfarin. In the presence of warfarin, vitamin K epoxide accumulates. (3) is an NADPH-dependent reductase which is relatively insensitive to the effects of warfarin. The carboxylase enzyme (4) is responsible for the γ -carboxylation of glutamine residues to Gla-residues (Holbrook *et al.*, 1996).

vitamin K-dependent clotting factors (II, VII, IX, and X) and for anticoagulants proteins C and S (Fig. 13.1) (Holbrook *et al.*, 1996; Suttie *et al.*, 1988). In the process of participating in the reaction responsible for adding Gla-residues to these factors, which occurs only in the liver, vitamin K is converted to vitamin K₁ 2,3 epoxide (vitamin K epoxide) (Bjornsson, 1984). The epoxide is normally recycled back to the reduced quinine form by vitamin K₁ epoxide reductase and then further reduced to the active vitamin, the hydroquinone, by an NADPH-dependent vitamin K quinine reductase (Choonara *et al.*, 1988). Warfarin exerts its anticoagulant effect by blocking the epoxide reductase enzymes in a dose dependent fashion, thereby interrupting the supply of functional vitamin K-dependent clotting factors and leading to accumulation of inactive vitamin K epoxide.

III. THERAPEUTIC USES OF VITAMIN K

Two to three million patients in the United States are receiving long-term anticoagulant therapy with warfarin (Libby and Garcia, 2002). Therapy with warfarin carries the benefit of prevention of thrombosis and the risk of hemorrhage. Published reports of annual bleeding risk have reported episodes of major bleeding to be as low as 1.3% per year in large, international trials of warfarin use to prevent stroke in atrial fibrillation (Ezekowitz *et al.*, 1992). The risk of hemorrhage is related to a number of factors, including increases in the INR, age, history of bleeding, renal

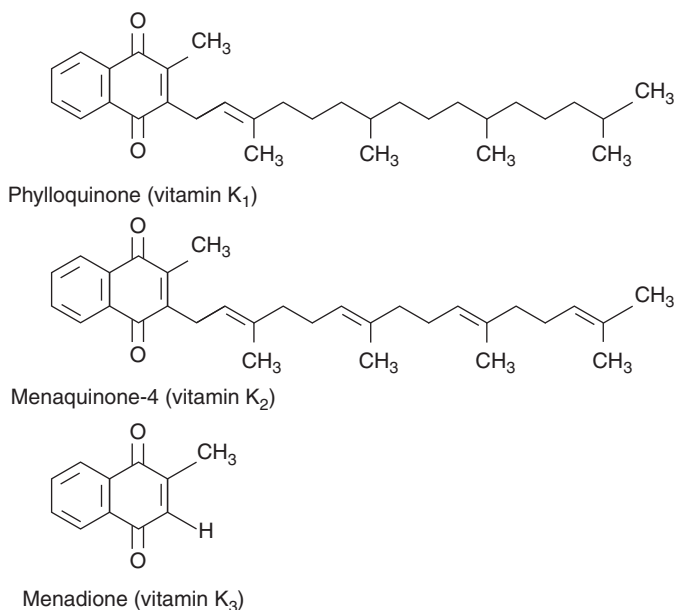


Figure 13.2 The vitamin K molecules (Martinez and Barsigian, 1998).

insufficiency, and a history of peptic ulcer disease (Hylek *et al.*, 2000). Bleeding risk increases dramatically as the INR rises above 4–5. Even brief episodes of time with elevated INRs pose a significant risk of bleeding for patients. Therefore, strategies that allow the practitioner to keep patients taking anticoagulants within minimizing the amount of time spent with an elevated INR should decrease the incidence of major hemorrhage related to warfarin use. Vitamin K (phytonadione) has been recognized for decades as a treatment for excessive anticoagulation. The American College of Physicians Consensus Conference (ACCP) on Antithrombotic therapy recommends the use of vitamin K₁ when the INR exceeds 6 (Ansell *et al.*, 2004). The American Heart Association, the American College of Cardiology, and the Anticoagulation Forum all endorse these recommendations for management of excessive anticoagulation.

Therapeutic options for correcting over-anticoagulation include temporarily interrupting warfarin therapy, administration of vitamin K₁, fresh frozen plasma (FFP), prothrombin complex concentrate (PCC), or very low dose recombinant factor VIIa.

There are two approaches in the management of hemorrhagic risk in patients anticoagulated with warfarin for any reason. The method chosen reflects the clinical seriousness of bleeding and balances against the thrombotic risk of a temporary discontinuation or reduction in the dose of warfarin. This latter area is important since patients may require the

reinstitution of warfarin for the underlying need for anticoagulation such as prosthetic heart valves, atrial fibrillation, or recurrent thromboembolic events.

IV. PATIENTS WITH MAJOR HEMORRHAGE

There is consensus that life-threatening bleeding requires rapid warfarin reversal regardless of the reason for anticoagulation. The use of FFP is commonly recommended in this clinical scenario but a number of considerations must be kept in mind. The concentration of factors II, VII, IX, and X is variable in each unit of FFP. The volume and the infusion duration of FFP must be considered in patients with limited cardiac reserve and urgency of reversing major bleeding. PCC, another option used for controlling patients with major hemorrhage, contains high concentrations of factors II, VII, IX, and X. Studies comparing the efficacy of FFP versus PCC have demonstrated a more rapid and effective reversal with PCC (Boulis *et al.*, 1999; Makris *et al.*, 1997). It should be noted that PCC is associated with thrombotic complications. Both FFP and PCC have reported infectious complications, but PCC is virally inactivated and is generally considered safer than FFP. The addition of intravenous vitamin K₁ concomitant with FFP or PCC adds to the reversal process by the endogenous synthesis of factors II, VII, IX, and X to the management. More recently, the use of recombinant human factor VIIa concentrate has been used in warfarin-associated major hemorrhagic events. In three small case series involving a total of 24 patients, recombinant factor VIIa dose (10–90 µg per kg of body weight) was successively given for the rapid reversal of elevated INR and bleeding complications (Deveras and Kessler, 2002; Lin *et al.*, 2003; Sorensen *et al.*, 2003). There have been reported thrombotic events associated with the use of recombinant factor VIIa. This agent is not recommended for prolong INR bleeding and is expensive, thus limiting its availability (Tables 13.3 and 13.4A,B) (Ansell *et al.*, 2004; Dentali *et al.*, 2006).

V. PATIENTS WITHOUT BLEEDING BUT PROLONGED INR

Several trials have looked at the safety and efficacy of oral, subcutaneous, and intravenous vitamin K₁ (phytonadione) administration for the correction of excessive anticoagulation. A retrospective review comparing the effectiveness of oral (2.5 mg or 5 mg), low dose intravenous (0.5 mg), high dose intravenous (1–10 mg), and subcutaneous (1–10 mg) vitamin K₁ administration determined that overcorrection (INR < 2.0) occurred most frequently in the high dose intravenous group, and undercorrection (INR > 5) was a greater problem in the subcutaneous group (Whitling *et al.*, 1998).

Table 13.3 American College of Physicians guidelines for managing elevated INRs in patients on warfarin therapy

INR	Bleeding	Management
INR < 5.0 [Level 2C]	No	Omit or lower warfarin dose More frequent INR monitoring
INR > 5.0 to < 9.0 [Level 2C]	No	Omit warfarin doses More frequent INR monitoring OR Omit warfarin doses Oral vitamin K 2.5–5.0 mg For patients at increased bleeding risk
INR > 9.0 [Level 2C]	No	Hold warfarin therapy Oral vitamin K 5–10 mg Frequent INR monitoring Use additional vitamin K as indicated
Any prolonged INR [Level 1C]	Major bleeding	Hold warfarin therapy Intravenous vitamin K, 10 mg PCC, FFP
Any prolonged INR [Level 1C]	Life-threatening bleeding	Hold warfarin therapy Intravenous vitamin K, 10 mg PCC, FFP, Recombinant factor VIIa Blood product support as needed

INR, International normalization ratio; PCC, Prothrombin Complex Concentrate. [Ansell et al., 2004](#).

Therefore, the low dose intravenous (0.5 mg) and oral (2.5–5 mg) vitamin K₁ routes of administration appear to be satisfactory alternatives. In a prospective controlled trial, 51 nonbleeding patients with an INR ranging from 4.5 to 10 received vitamin K 1.0 mg subcutaneous or orally in addition to having warfarin held ([Crowther et al., 2002](#)). Oral vitamin K₁ administration normalized the INR more rapidly than did subcutaneous administration. Comparison of oral (2.5 mg) versus intravenous (0.5 mg) vitamin K₁ administration in 44 patients with baseline INR 6–10 without major bleeding showed that a therapeutic range (INR 2–4) at 6–12 h was reached more rapidly for the intravenous than the oral vitamin K₁ group ([Lubetsky et al., 2003](#)). At 24 h, the mean INR values were similar for the oral and intravenous groups. From these clinical trials, it is evident that oral administration of vitamin K produces major corrections in INR at 24 h. Compared to the subcutaneous route, oral therapy reverses anticoagulation more rapidly although not faster than the intravenous route. The exact dose of oral vitamin K is 1.0–2.5 mg, which is appropriate for an INR between 4 and 10. Clinical trials have compared the

Table 13.4A Treatment strategies for prolonged INRs without bleeding in patients on warfarin therapy

INR 4.5–10	1. Withhold warfarin therapy 2. Administer 1 mg, PO, vitamin K 3. Reintroduce warfarin at a lower dose on the following day, recheck INR in <72 h 4. Identify and correct the etiology for the prolonged INR
	OR
INR > 10	1. Withhold warfarin, recheck INR in 24–48 h 1. Withhold warfarin 2. Administer 2.5 mg or 5.0 mg, PO, vitamin K 3. Recheck INR in 24 h
	OR
	1. Withhold warfarin, administer 0.5–1.0 mg, IV, vitamin K 2. Recheck INR in 24 h

INR, International normalization ratio; PO, By mouth. [Dentali et al., 2006](#).

Table 13.4B Treating strategies for any prolonged INR with major bleeding in patients on warfarin therapy

Major but not life-threatening Bleeding with any prolonged INR	1. Withhold warfarin 2. Administer vitamin K 1 mg, IV 3. Plasma complex concentrate 4. FFP 5. Define etiology of bleeding 6. Blood product support: PRBC, platelets
Life-threatening bleeding With any prolonged INR	1. Withhold warfarin 2. Administer vitamin K 1 mg, IV 3. Plasma complex concentrate 4. FFP 5. Blood products support: PRBC, platelets 6. (rFVIIa)

[Dentali et al., 2006](#).

FFP, Fresh Frozen Plasma; rFVIIa, Recombinant factor VIIa; IV, Intravenous; INR, International normalization ratio; PRBC, Packed Red Blood Cells.

intravenous versus subcutaneous route of vitamin K₁ administration. Fifty-five asymptomatic subjects were randomized to receive either subcutaneous or intravenous 0.5 mg vitamin K₁ for baseline INR values of 6–10 and 3 mg

vitamin K₁ for baseline INR values of 10–20 (Nee *et al.*, 1999). At 24 h, the mean INR was significantly lower in all subjects receiving intravenous vitamin K₁ compared to subcutaneous vitamin K₁; however, at 72 h, the mean INR in all subjects was similar in both groups. The results of the study suggest that compared to the subcutaneous route of administration, the intravenous route results in prompt reduction in INR. In another study, a direct comparison of the intravenous (1 mg) and subcutaneous (1 mg) route of administration of vitamin K was evaluated in 22 asymptomatic patients with mean INR of 6–8.5 (Raj *et al.*, 1999). A more rapid correction of the INR was seen with the intravenous route (3.1) than with the subcutaneous route (5.0; $P = .009$). The optimal dose of intravenous vitamin K₁ appears to be 0.5–1.0 mg. Higher doses may be needed or a repeat dose required if target INR reversal goals are <2.0 or large amount of warfarin were taken (Tables 13.3 and 13.4A) (Ansell *et al.*, 2004; Dentali *et al.*, 2006).

The incidence of anaphylaxis with vitamin K₁ was recently evaluated in a retrospective review in which over 58 months, a total of 6572 doses of intravenous vitamin K₁ infusion were given (Riegert-Johnson and Volcheck, 2002). Two cases of anaphylaxis were reported, leading to an incidence of 3 per 10,000 doses (95% CI = 0.04–11 per 10,000 doses). The authors also identified 14 cases of intravenous vitamin K₁ anaphylaxis in the literature. The anaphylaxis reaction may be related to the solubilizing vehicle; polyethoxylated castor oil (PEO-CO) vehicle does not seem to be the sole cause of the adverse event since cases of anaphylaxis have been reported with formulation of the intravenous vitamin K₁ that do not contain the PEO-CO vehicle.

VI. NEW PHYSIOLOGIC FUNCTIONS OF VITAMIN K

After the discovery of Gla-residues in prothrombin, the subsequently identified Gla-proteins were those involved in blood coagulation: Factor VII, IX, and X as well as the proteins C, S, and Z. Carboxylase was demonstrated in a wide variety of nonhepatic tissues, ranging from bone to skin (de Boer-van den Berg *et al.*, 1986; Lian and Friedman, 1978). It was assumed that the endogenous substrate of carboxylase in these tissues was not involved in blood coagulation. The first extrahepatic sites in which Gla-proteins have been identified and characterized are bone and cartilage. Bone Gla-protein is called osteocalcin, which serves as a marker for osteoblastic activity (Hauschka *et al.*, 1975; Price *et al.*, 1976). Matrix Gla-protein (MGP) is the strongest inhibitor of tissue calcifications known. In humans, loss of function mutations of MGP result in the Keutel syndrome characterized by abnormal cartilage calcification (Munroe *et al.*, 1999). In 1971,

Keutel *et al.* described the syndrome in two siblings presenting with abnormal cartilage calcification, brachytelephalangism, neural hearing loss, and peripheral pulmonary stenosis (Keutel *et al.*, 1971). In healthy tissues, the expression of MGP is low but in areas of arterial calcification MGP levels are elevated, which may be triggered by calcium crystals (Schurgers *et al.*, 2001). This elevation of MGP may serve as a marker of cardiovascular disease in the near future (Schurgers *et al.*, 2005). Recently, another extrahepatic Gla-protein was identified and named growth-arrest-specific gene 6 (Gas6). Gas6 exhibits a broad range of regulatory functions associated with cell growth regulation, migration and proliferation, cell survival, apoptosis, recognition of dying cells, phagocytosis, and cell adhesion (Hafizi and Dahlback, 2006). Gas6 serves as a ligand for a subfamily of receptor tyrosine kinases, and it has been demonstrated that incomplete carboxylation results in loss of biological activity (Nakano *et al.*, 1997).

VII. WARFARIN EFFECTS ON VITAMIN K

Vitamin K-dependent Gla-proteins regulate not only blood coagulation but also other physiological processes including bone metabolism (osteocalcin), vascular calcification (MGP), cell growth and apoptosis (Gas6) and that as a consequence oral anticoagulants may have undesired effects in extrahepatic tissue.

Two recent studies examined the chronic use of warfarin and the risk of fracture in older women. One study reported no association between long-term warfarin treatment and fracture risk, while another found a significantly higher risk of rib and vertebral fractures in warfarin users compared to nonusers (Caraballo *et al.*, 1999a; Jamal *et al.*, 1998). A meta-analysis of the results of 11 published studies found that oral anticoagulation therapy was associated with a very modest reduction in bone density at the wrist, and no change in bone density at the hip or spine (Caraballo *et al.*, 1999b).

Infrequently, daily supplemental vitamin K₁ has been clinically used in patients receiving warfarin therapy in order to control fluctuating INR levels (Reese *et al.*, 2005; Sconce *et al.*, 2007). Since the level of anticoagulation is a balance between warfarin and dietary vitamin K, introducing vitamin K₁ supplementation will require concordant warfarin adjustment. This is a very challenging clinical management scenario. Cranenburg *et al.* commented on this approach with the major concern that supplemental vitamin K₁ would result in an increased dosing of warfarin, which would exhaust the vitamin K stores in the arterial walls (Cranenburg *et al.*, 2007). The focus of this hypothesis was demonstrated in rats receiving vitamin K₁ plus warfarin therapy. They developed widespread arterial calcifications within 2–4 weeks (Price *et al.*, 1998). Recent research in this area has

demonstrated that vitamin K₂ (MK-4 and MK-7) is equally transported to the liver and extrahepatic tissues. The use of vitamin K₂ in patients receiving warfarin would require that the MK-7 form, which has a 3 day half-life, and reach a steady level in 2 weeks (Schurger *et al.*, 2007). A trial has been proposed using vitamin K₂ (MK-7) in patients on warfarin therapy. The stability of their INR value over time, bone density, and vascular calcification will be the endpoints of the trial (Cranenburg *et al.*, 2007; Stafford *et al.*, 2007).

VIII. SUMMARY

Since its discovery in the 1930s, vitamin K has demonstrated its effectiveness as a cofactor for the posttranslational carboxylation of glutamate residues, which is essential for the formation of the liver-generated clotting factors II, VII, IX, X, protein C, and protein S. A number of vitamin K-deficient disease states have been identified and successfully treated over the past 60 years. The discovery of warfarin and its widespread use in the past 20 years in patients with atrial fibrillation, prosthetic heart valves, deep vein thrombosis, and pulmonary embolism has created a new use for vitamin K. National guidelines now recommend vitamin K dosing schedules for various degrees of prolonged INRs. In the past decade other extrahepatic sites for Gla-protein activity have been defined, which include bone, liver, and cells. These new discoveries will affect bone metabolism and arterial health as well as prevent warfarin-induced side effects. Ongoing and new research in the field of vitamin K metabolism are in progress and will reveal new insights into this vitamin's essential functions.

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CONGENITAL BLEEDING DISORDERS OF THE VITAMIN K-DEPENDENT CLOTTING FACTORS

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Abstract

Congenital bleeding disorders of the vitamin K-dependent coagulation factors represent only about 15–20% of all congenital bleeding disorders. However, they played an important role of the history of blood coagulation.

Prothrombin was the first entity dealt with. Subsequently, in the late 1940s or early 1950s, the discovery of factor IX allowed the separation of hemophilia into two groups, A and B. In the 1950s, the discovery of factors VII and X allowed the formulation of a logic and plausible explanation for the clotting mechanism. The subsequent discovery of vitamin K-dependent proteins with an inhibitory effect on blood coagulation has further enhanced the importance of the vitamin K-dependent clotting factors.

Recently, the study of families with multiple defects of the prothrombin complex has spurred the interest in vitamin K metabolism and the γ -carboxylation system. The relevance of these studies had also an important role in the understanding the mechanism of action of other noncoagulation-related proteins. The vitamin K-dependent clotting factors represent a homeostatic mechanism at the basis of the hypercoagulability (thrombosis)—hypocoagulability (hemorrhagic) system, namely, to a mechanism that is vital for survival.

The different bleeding condition will be dealt with separately, namely, prothrombin or Factor II, Factor VII, Factor IX (hemophilia B), and Factor X deficiencies. An additional heading deals with the combined defect of the prothrombin complex, namely, combined deficiency of Factor II, Factor VII, Factor IX, and Factor X. Since, sometimes, a hemorrhagic role has been attributed to Protein Z deficiency, another vitamin K-dependent protein, this defect will also be dealt with, even though briefly.

Each deficiency has been approached in a global manner, namely, with adequate reference to history, background, prevalence, classification, hereditary pattern, biochemistry and function, molecular biology, clinical picture, updated laboratory diagnosis, prognosis, and therapy. Particular emphasis has been placed on the significance of cases with “true” deficiency [cross-reacting material (CRM negative)] and cases with abnormalities (CRM positive). The genetic, clinical, and laboratory implications of these two forms have been extensively discussed in every instance. The importance of a multiple, combined diagnostic approach that has to include whenever possible clotting, chromogenic, immunological, and molecular biology studies has been underlined. Clotting tests have to be carried out using different activating agents since results may vary, thereby indicating a different reactivity of the abnormal protein. Molecular biology techniques, alone, are unable to supply plausible diagnostic conclusions. In fact the genotype–phenotype relation has not been clarified so far for most of these bleeding conditions. Recent progress in management such as the use of recombinant factor concentrates, results of liver transplantation, and attempts at genetic therapy has been discussed. Potential complications of therapeutic measures have also been discussed.

A section dealing with future putative aims of research in this field will close the chapter. © 2008 Elsevier Inc.

I. INTRODUCTION

Bleeding disorders of the prothrombin complex (PC) factors, namely, Factor II, Factor VII, Factor IX, and Factor X, represent an important part of the history of blood coagulation (Greenberg and Davie, 2006). One of them, Factor IX, responsible for hemophilia B was suspected when it was shown about 60 years ago that a hemophilic plasma was corrected by the addition of the plasma of another patient with “hemophilia” (Livingstone and Johnstone, 1963). This observation allowed to separate the hemophilia in two groups, A and B (Aggeler *et al.*, 1952; Biggs *et al.*, 1952). Prothrombin deficiency was the first coagulation defect, after hemophilia, to be known and studied as the source of thrombin.

Factor VII deficiency was described in 1951 by separating it from prothrombin deficiency (Alexander *et al.*, 1951).

Factor X deficiency was discovered in 1956–1957, and in this case too the defect was observed in one family by restudying a patient originally described as “hypoproconvertinemia” (Bachmann *et al.*, 1957; Hougie *et al.*, 1957; Kemkes-Matthes and Matthes, 2001; Telfer *et al.*, 1956).

These factors have a common origin even though a different genetic control. In fact, Factors II, VII, and X are controlled by autosomal recessive genes, whereas Factor IX deficiency (hemophilia B) has an X-linked hereditary pattern. As a consequence, both sexes are affected in cases of Factor II,

Factor VII, and Factor X deficiencies, but only males are affected by hemophilia B. Exceptional description of hemophilia B in women is due to the mating of a carrier and a hemophiliac or to extreme lyonization or to rare chromosome X abnormalities (Shetty *et al.*, 2001a). The phylogenetic significance of this discrepancy is unknown, if any.

Different chromosomes are involved in the control of the synthesis of these factors.

X chromosome is involved in hemophilia B, whereas chromosome 13 controls Factor VII, Factor X, and Protein Z. Factor II depends on genes located in chromosome 11; for comparison the other two PC factors, Protein C and Protein S are under control of genes located in chromosomes 2 and 3, respectively. The significance of these different controls is not fully understood. The close location on the long arm of chromosome 13 of Factor VII, Factor X, and Protein Z genes suggests a common origin.

The PC factors have similar biochemical features; they are vitamin K-dependent glycoproteins but quite different biological half-life, very short for Factor VII, very long for Factor II, while those for Factors IX and X are in between that range. Note that the half-life of coagulant or anticoagulant factor PC has some similarities. For example, two of them, FVII and Protein C, have short half-life, whereas others, for example Protein S and Factor II, have long ones. Does this possible pairing have any significance? We do not know.

The clinical manifestations of all these deficiencies are similar and involve all structures with a prevalence of hemarthrosis for Factor IX deficiency. Brain hemorrhage is a frequent cause of death in all cases. It has been described in fact not only in Factor VII deficiency but also in all other conditions.

In general, the deficiency of these factors presents a variable gravity of bleeding manifestations that, with the exception of Factor VII deficiency, correlates fairly well with the clotting activity of the factor involved.

Factor IX and Factor VII are the least rare, whereas Factor II and Factor X are very rare coagulation disorders.

The classification of coagulation defects has become, in recent years, more difficult because of the several variants described. An important element for Factor II, Factor VII, and Factor X is the distinction between cases of "true" deficiency (concomitant decrease of activity and antigen, namely, cross-reacting material—CRM—negative cases or Type I) and abnormalities (low activity with variable, but always significantly higher, antigen levels, namely, CRM positive cases or Type II).

The classification for Factor IX is similar with the exception that some of CRM positive patients also possess a peculiar inhibitory action (hemophilia Bm) on the Tissue Factor + Factor VII system. Such inhibitory behavior has never been described for the CMRpos cases of the other clotting factors. Homozygotes for Factor II or Factor VII or Factor X deficiencies and compound or double heterozygotes for two deficiencies (hypo-hypo)

have usually activity and antigen levels between 1% and 10% of normal. Factor activity above 10% of normal is usually associated with a mild spontaneous bleeding tendency, but bleeding may be severe after surgery or trauma. Heterozygotes with factor activity levels around 50% of normal are only occasionally symptomatic.

Occasional excessive bleeding especially after surgery or trauma has been described for heterozygotes for Factor II or Factor X. Heterozygotes for Factor VII deficiency are asymptomatic.

Factor IX deficiency (hemophilia B) is subdivided, as hemophilia A, in severe (Factor IX, <1%), moderate (Factor IX, 1–5%), and mild (Factor IX, 6–30%). Bleeding intensity correlates well with factor activity levels. Carriers of hemophilia B have activity level about 50% of normal and are asymptomatic.

It is puzzling that three of the four hemorrhagic factors are under autosomal genes, whereas the remaining one is an X-linked trait. This aspect, together with other major features of the PC factors, is gathered in [Table 14.1](#).

The role played by PC in evolution is unknown.

The dependence on diet (vitamin K intake) for all these factors may have a biological significance. In subjects with poor diets, the concomitant decrease of both clotting and inhibitory factors assured a downgraded hemostatic balance.

In coumarin drugs treatment, the coagulation pattern favors hypocoagulation. However, the occasional thrombotic manifestation seen in coumarin-treated patients indicates that this hypocoagulable state sometimes fails.

We will describe the bleeding disorder of all PC factors separately. The defect that causes the concomitant decrease of all PC clotting factors will be dealt with separately, since it is a distinct clinical entity.

Combined defects of Factor II, or Factor VII, or Factor IX or Factor X with other clotting factors, will be dealt with after the description of the single deficient states.

Protein Z deficiency, even though not surely linked with a bleeding tendency, will be dealt with only briefly. Needless to say that the prothrombotic defects of the PC will not be dealt with being outside the scope of this chapter.



II. CONGENITAL PROTHROMBIN (FACTOR II) DEFICIENCY

Congenital bleeding defects of prothrombin are one of the rarest coagulation disorders. In 1975, a preliminary search carried out by us gathered only 25 cases ([Girolami *et al.*, 1975b](#)). A more recent study in 1999 gathered about 25 cases of proven or probable hypoprothrombinemia

Table 14.1 Main features of prothrombin-complex factors

	MW	Controlling genes in chromosome	Hereditary pattern	Estimated prevalence	Approximate concentration (mg%)	Half- life (hours)	Main function	Comments
Factor II	72,000	11	AR	1:2,000,000	~10.0	~70	FXa substrate	
Factor VII	50,000	13	AR	1:500,000	~0.1	~5	TF cofactor	
Factor IX	57,000	X	XL	1:30,000	~0.4	~20	Part of prothrombinase	
Factor X	59,000	13	AR	1:1,500,000	~1.0	~30	FII activator	
Protein Z	62,000	13	AR ?	?	~0.25	~30	Unknown	Hemorrhagic effect not proven. Prothrombotic role not clear
Protein C	62,000	2	AR	1:100,000 ?	~0.4	~7	Inhibitor	
Protein S	69,000	3	AR	1:100,000 ?	~0.2	~40	Inhibitor cofactor	

Besides data for bleeding disorders, comparative data are also supplied for prothrombotic vitamin K-dependent proteins. Figures are only orientative and, often, approximate.

AR, autosomal recessive; XL, X-linked; MW, molecular weight; TF, tissue factor.

and about 40 cases of dysprothrombinemia (Girolami *et al.*, 1999a). Since that time, a few additional cases have been reported. The first reports on patients with hypoprothrombinemia go back to the studies by Quick (Quick, 1947; Quick and Hussey, 1962; Quick *et al.*, 1955).

Because of the lack of adequate antiprothrombin antiserum, the cases were thought to be cases of true deficiency, namely, hypoprothrombinemia. Subsequently, it was shown that these two patients were cases of dysprothrombinemia (Henriksen and Mann, 1988, 1989). On the contrary, the family studied by van Creveld (van Creveld, 1954) was subsequently demonstrated to be cases of true deficiency (Hart, 1965; Poort *et al.*, 1994). This restudy of old reports with a consequent different allocation to the dysforms complicates the evaluation of the literature.

In 1969, Shapiro *et al.* (1969) described the first family with dysprothrombinemia (Prothrombin Cordeza). In these patients, and in a few others subsequently reported, there was a discrepancy between clotting activity and antigen. Reviews of these variants were published in 1996 and 1999 (Collados *et al.*, 1996; Girolami *et al.*, 1999a).

The fact that even in severe cases, traces of prothrombin are found in the plasma of the patients, seems to indicate that the complete absence of prothrombin is incompatible with life. At this point, it may be relevant to note that even complete absence of antithrombin is incompatible with life.

A. Classification and prevalence

Prothrombin defects can be classified as other defects of clotting factors in:

1. True deficiency (hypoprothrombinemia) (homozygotes and heterozygotes)
2. Dysprothrombinemia (homozygotes and heterozygotes)
3. Hypo-dys or dys-dys forms (compound heterozygotes)
4. Combined or multiple defects

True prothrombin deficiency is probably the rarest coagulation disorder.

The hallmark of this defect is the concomitant decrease of both prothrombin activity and antigen. The discrepancy is usually of a few percentile figures and should be by the definition of <10%. A few of the cases listed as cases of hypoprothrombinemia have not yet been studied immunologically, so their allocation in the group of hypoprothrombinemia remains questionable. Usually no eponym is used to describe cases of hypoprothrombinemia (Table 14.2) (Baudo *et al.*, 1972; Borchgrevink *et al.*, 1959; Catanzarite *et al.*, 1997; Crossley *et al.*, 1992; De Bastos *et al.*, 1964; Degen *et al.*, 1995; Gill *et al.*, 1978; Girolami, 1971; Ikkala *et al.*, 1971; Josso *et al.*, 1962; Kattlove *et al.*, 1970; Lee and Li, 2002; Lefkowitz *et al.*, 2003; Livingstone and Johnstone, 1963; Montgomery *et al.*, 1978; Pina-Cabral and Justica,

Table 14.2 Cases of homozygous prothrombin “true” deficiency reported in the world literature

References	No. of cases	Sex	Parents consanguinity
Hart <i>et al.</i> (1965), Poort <i>et al.</i> (1994), and van Creveld (1954)	6	4 M; 2 F	No
Borchgrevinck <i>et al.</i> (1959)	1	F	Yes
Josso <i>et al.</i> (1962)	1	M	Yes
Pool <i>et al.</i> (1962)	1	M	No
Livingstone and Johnstone (1963)	1	M	?
De Bastos <i>et al.</i> (1964)	3	2 M; 1 F	Yes
Girolami <i>et al.</i> (1971)	1	F	Yes
Kattlove <i>et al.</i> (1970)	2	1 M; 1 F	?
Girolami <i>et al.</i> (1970)	1	M	Yes
Girolami <i>et al.</i> (1970)	1	M	No
Ikkala <i>et al.</i> (1971)	1	F	No
Baudo <i>et al.</i> (1972)	1	M	Yes
Piña-Cabral <i>et al.</i> (1973)	1	M	No
Gill <i>et al.</i> (1978) and Tamary <i>et al.</i> (1997)	1	F	?
Montgomery <i>et al.</i> (1978)	1	M	?
Catanzarite <i>et al.</i> (1997)	1	F	No
Poort <i>et al.</i> (1997)	1	F	No
Sun <i>et al.</i> (1999) ^a	1	F	Yes
Lee and Li (2002)	1	M	?
Lefkowitz <i>et al.</i> (2003)	4	3 M, 1 F	?
Wang <i>et al.</i> (2004)	1	F	Yes

The list may be incomplete.

^a Patient has same mutation as that previously reported by Poort *et al.* (1994), namely, Tyr 44Cys.
F, female; M, male.

1973; Pool *et al.*, 1962; Poon, 1981; Poort *et al.*, 1994, 1997; Rubio *et al.*, 1983; Strijks *et al.*, 1999; Valls-de-Ruiz *et al.*, 1987).

Dysprothrombinemia are usually indicated according to the city or area where they were first described. A few are unnamed or a tentative definition has been attributed by us for the sake of completion (Akhavan *et al.*, 2003; Bezeaud *et al.*, 1984, 1979; Board *et al.*, 1982; Board and Shaw, 1983; Dumont *et al.*, 1987; Friezner Degen *et al.*, 1995; Girolami *et al.*, 1974a, 1977, 1978; Henriksen and Mann, 1988; Henriksen *et al.*, 1998; Huisse *et al.*, 1985; Iwahana *et al.*, 1992; James *et al.*, 1994, 1995; Josso *et al.*, 1971, 1982; Kahn and Govaerts, 1974; Lefkowitz *et al.*, 2003; Miyata *et al.*, 1987, 1995; Montgomery *et al.*, 1980; Morishita *et al.*, 1992; O'Marcaigh *et al.*,

1996; Owen *et al.*, 1978; Poon *et al.*, 1979, 1981; Rocha *et al.*, 1986; Ruiz-Saez *et al.*, 1986; Sabharwal *et al.*, 1992; Shapiro *et al.*, 1973; Smith *et al.*, 1981; Sun *et al.*, 2001; Tamary *et al.*, 1997; Weinger *et al.*, 1980).

The hallmark of these conditions is a discrepancy between clotting activity and antigen level. The latter is always higher than the clotting counterpart and sometimes perfectly normal (Akhavan *et al.*, 2003).

Several families with a sufficiently proven prothrombin abnormality have been described so far. As a matter of fact, dysprothrombinemias seem more frequent than true hypoprothrombinemia do. Most of these conditions appear to be distinct entities. However, some appear to be identical to conditions previously described under different eponyms. For example, prothrombin Dhahran seems identical to prothrombin Padua, and therefore they should be dealt with together. The same is true for prothrombin Tokushima and prothrombin Molise. Furthermore, prothrombin Frankfurt seems identical to prothrombin Salakta. Finally, prothrombins Madrid and Obihiro seem identical to prothrombin Barcelona. Since prothrombins Padua, Molise, Salakta, and Barcelona were originally described before Dhahran, Tokushima, Frankfurt, Madrid, or Obihiro, respectively, the first eponym will be used to describe these abnormalities, unless different mutations are demonstrated.

Prothrombin Mexico City could be excluded from the dysprothrombinemia group and be included among the hypoprothrombinemias, because of the concomitantly low levels (about 10%) of the antigen and activity observed in this deficiency. The same is true for prothrombin Philadelphia which is characterized by 8% activity and 4% antigen level. This picture, by definition, is not compatible with the basic features of an abnormality (Table 14.3).

Prothrombin abnormalities may be, tentatively, classified according to the site of the defects as follows: defect in the activation mechanism or defects in the thrombin molecule. As is discussed later, the latter group can be subdivided into two subtypes, namely, a first subtype in which there is an abnormal interaction of thrombin with fibrinogen and a second one with a defective catalytic activity.

Molecular biology analysis has allowed further classification of the defect, even though an exact genotype–phenotype relation is not completely clear yet.

B. Biochemistry

Human prothrombin is a glycoprotein with a molecular weight of about 70,000 Da, and with a known amino acid composition. It is synthesized at the hepatocyte level in the presence of vitamin K. The plasma concentration is about 10 mg%, and the biological half-life is about 70 h (Friezner Degen, 1995; Shapiro and McCord, 1978). The circulating mature protein is a

Table 14.3 Cases of prothrombin abnormalities described in the world literature

References	Eponym	Type of defect	Sex	Parents consanguinity
Quick <i>et al.</i> (1947, 1955), Quick and Hussey (1962), Owen <i>et al.</i> (1978), and Henriksen and Mann (1988, 1989)	Quick I, II	Comp. Het.	1 M; 1 F	?
Shapiro <i>et al.</i> (1969)	Cardeza	Het.		?
Josso <i>et al.</i> (1971)	Barcelona	Hom. and Het.	4 M; 2 F	No
Girolami <i>et al.</i> (1974)	Padua	Het.	4 M; 1 F	No
Kahn and Govaerts (1974)	Brussels	Hom. and Het.	4 M; 1 F	Yes
Shapiro <i>et al.</i> (1973)	San Juan	Comp. Het.	5 M; 2 F	?
Girolami <i>et al.</i> (1978b)	Molise	Comp. Het.	1 M; 1 F	Yes
Poon <i>et al.</i> (1979, 1981)	Birmingham I and II	?	?	?
Montgomery <i>et al.</i> (1980) and Lefkowitz <i>et al.</i> (2003)	Denver	Hom. and Het.	2 M; 1 F	No
Weinger <i>et al.</i> (1980)	Houston	?	1 M	?
Smith <i>et al.</i> (1981)	Gainesville	Het.	2 F	?
Josso <i>et al.</i> (1982)	Metz	Comp. Het.	3 M; 9 F	?
Rubio <i>et al.</i> (1983)	Habana	Comp. Het.	3 M; 1 F	?
Board and Shaw (1983)	Canberra	?	?	?
Bezeaud <i>et al.</i> (1984)	Salakta	Hom.?	1 M; 1 F	?

(continued)

Table 14.3 (continued)

References	Eponym	Type of defect	Sex	Parents consanguinity
Huisse <i>et al.</i> (1985)	Clamart	Het.	2 F	No
Rocha <i>et al.</i> (1986) and Akhaven <i>et al.</i> (1999)	Segovia	Hom. and Het.	2 M; 1 F	Yes
Ruiz-Saez <i>et al.</i> (1986) and Sekine <i>et al.</i> (2002)	Perija	Hom. and Het.	2 M; 2 F	Yes
Dumont <i>et al.</i> (1987)	Poissy	Hom. and Het.	2 M; 2 F	Yes
Lutze <i>et al.</i> (1989)	Magdeburg	Het.	4 M; 3 F	?
Morishita <i>et al.</i> (1992)	Himi	Comp. Het.	1 M; 3 F	No
Strijks <i>et al.</i> (1999)	Nejmegen	Hom. and Het.	1 M	Yes
Akhavan <i>et al.</i> (2000) ^a	G	Hom.	1 M	No
	I	Hom.	1 F	Yes
Akhavan <i>et al.</i> (2000) ^a	A	Hom.	1 F	Yes
	B, C1, C2	Comp. Het.	2 F, 1 M	Yes
	D	Hom.	1 F;	No
	E, F	Hom.	1 F, 1 M	Yes, No
	H	Hom.	1 F	No
	J1, J2, K	Hom.	1 F, 2 M	No
Lefkowitz <i>et al.</i> (2003)	Puerto Rico 1	Hom.	1 M; 1 F	Yes
	Puerto Rico 2	Hom.	n.r.	Yes

The list may be incomplete.

Prothrombins Corpus Christi, Madrid and Obihiro, Daharan, Tokushima, and Frankfurt are not listed, since they seem identical to prothrombins Quick I, Barcelona, Padua, Molise, and Salakta, respectively. Prothrombins Mexico City and Philadelphia have not been listed because they appear cases of true deficiency (concomitant decrease of activity and antigen). Het., heterozygotes; Hom., homozygotes; Comp. Het., compound heterozygotes; M, male; F, female; n.r. not reported.

^a Kinders indicated with letters as reported by authors.

single chain glycoprotein of 579 residues (Friezner Degen, 1995; Lanchantin *et al.*, 1968; Shapiro and McCord, 1978).

The prothrombin molecule contains five domains: the propeptide (residues -43 to -1), the Gla domain (residues 1-40), a kringle domain

(residues 41–155), a kringle 2 domain (residues 156–271), and a serine protease domain precursor (residues 272–579). Prothrombin is activated by Factor Xa on the surface of activated platelets in the presence of Factor V. Fragment 1–2, a large activation peptide, is thus obtained as the result of the cleavage of the peptides Arg 271–Thr 272. The remainder of the molecule is not yet enzymatically active and is termed Prothrombin 2. The second cleavage splits the prothrombin molecule at residue 320, forming a light and a heavy chain of thrombin without releasing them as separate entities because of a single disulfide bond. This two-chain molecule is α -thrombin, the active enzyme.

Other intermediate products are formed during the conversion of prothrombin into thrombin. The type and the relative presence of these intermediate products vary with the type of activation. The analysis of such activation pattern is beyond the scope of this chapter.

From a biochemical standpoint, congenital dysprothrombinemias could be classified into two groups, namely, defects in the activation mechanism; and defects in the thrombin molecule. The first group includes prothrombins San Juan (inability to bind Ca^{2+}), Barcelona (defective cleavage by FXa at site Arg 271–Thr), and Clamart (defective cleavage by FXa at Arg 321–Ile). In these cases and other similar dysprothrombinemias, prothrombin activation is abnormal and slow but thrombin, once formed, appears to be normal.

In the second group, the defect resides not in the activation mechanism but in the final product, namely, thrombin. As far as this type is concerned, two subtypes are usually recognized, one in which the defect lies in the interaction of thrombin with fibrinogen and the other in which there is a defect in the catalytic region of thrombin. Examples of the first subtype (defective interaction with fibrinogen) are: prothrombins Quick, Salakta, and Himi. Examples of abnormality subtype 2 (defective catalytic region) are prothrombins Molise (Tokushima), Habana, and Metz ([Table 14.4](#)).

C. Genetics and hereditary transmission

The gene encoding prothrombin is located on chromosome 11 (p11–p12). The gene contains 14 exons and 13 introns ([Degen and Davie, 1987](#)). The introns serve to separate the domains of the protein. Exons 1 and 2 encode the propeptide; the Gla domain is encoded by Exon 2 and is composed of the 10 glutamic acid residues that are carboxylated by vitamin K-dependent enzymes. These residues play a fundamental role in Ca-binding on cellular membranes.

Kringle 1 and 2 domains are encoded by Exons 3–6 and 7–8, respectively. The function of these domains is still unclear, even though it seems that kringle 2 is involved in the binding of Factor Xa.

Table 14.4 Prothrombin activity and antigen in the inherited dysprothrombinemias reported in the world literature

Abnormality	Activity (% of normal)	Antigen (% of normal)	Nature of defect	Bleeding
Cardeza	50	100	Prethrombin 2 region	No
Barcelona (Madrid, Obihiro)	12 (3,18)	100 (100, 105)	Factor Xa cleavage	Yes
Padua (Dhahran)	50 (6)	100 (100)	Factor Xa cleavage	Yes
Brussels	46	88	?	Yes
San Juan	20	93	Calcium-binding site	Yes
Molise (Tokushima)	11 (11)	45 (42)	Thrombin region- reduced interaction with fibrinogen	Yes
Quick I and II	<2	34	Thrombin region	Yes
Birmingham I, II	8–10 (<3)	24–30 (8–14)	?	Yes
Denver	<1 (5)	13 (21)	?	Yes
Houston	5–9	51	Factor X cleavage	Yes
Gainesville	23	70	Factor Xa cleavage	Yes
Metz	10	50	Thrombin region	Yes
Canberra	?	?	Pro-region	No
Salakta (Frankfurt)	16 (13)	100 (91)	Thrombin region	No (Yes)
Segovia	7–20	100	Fragment 2 defect?	Yes
Perija	2	70	Factor Xa cleavage	Yes
Clamart	50	100	Factor Xa cleavage	No
Poissy	2	47	?	Yes
Habana	10	50	?	Yes
Magdeburg	44	90	?	Yes
Himi I and II	10	88	Thrombin region	Yes
Kindred G	5–23	71	Thrombin region	Yes
Kindred I	1	71	Thrombin region	Yes
Puerto Rico, Kindred 3	9	35	Activation	Yes
Kindreds 4 and 5	15–19	28–40	Activation	Yes

The list may be incomplete.

Prothrombin Corpus Christi is identical to prothrombin Quick I.

Numbers or letters indicate kindreds as reported by authors. Levels are approximated to the closest number.

The thrombin- α chain, which is formed after FXa cleavage at 271–272 and 320–321 residues, is encoded by Exons 8 and 9 (catalytic area), while the β chain is encoded by Exons 9–14.

The overall evaluation of all mutations described in prothrombin deficiency and abnormalities does not allow yet a satisfactory comprehension of genotype–phenotype and of structure–function relations.

Prothrombin deficiency is transmitted as an autosomal recessive trait. The same is true for dysprothrombinemias. Compound defects are characterized by an association of a heterozygous “true” deficiency and a heterozygous abnormality, or by two, distinct abnormalities. These conditions are known as compound heterozygosis.

It is interesting to note a clear prevalence of patients with a Latin background. About 70% of all patients with prothrombin defects have come from countries of Latin background (Cardeza, Barcelona, Padua, Segovia, San Juan, Habana, Puerto Rico, etc.). The significance of this observation remains to be clarified (Fig. 14.1).

Recently, the genetic analysis of cases of true hypoprothrombinemia has been carried out. The large family originally described by van Creveld was shown to have a missense mutation from Tys 44 to Cys. The reason why this point mutation is associated with very low levels of both prothrombin activity and antigen remains to be clarified. At present, several other cases of hypoprothrombinemia were investigated by gene analysis as is shown in Table 14.5. Mutations involving several exons have been described. Most frequently they involve the catalytic area (Exons 8–14). A clear genotype–phenotype relation has not always been consistent. All these studies indicate the great heterogeneity of human prothrombin defects. In fact, mutation in different exons may cause similar CRMneg forms.

Several dysprothrombinemias have also been investigated from a molecular point of view. The different mutations seen in these dysprothrombinemias are gathered in Table 14.6. Occasionally, the same mutations have been found in prothrombin, originally labeled with different eponyms. It is clear that, until it is proven otherwise, the abnormalities have to be considered identical. Again, it is evident that mutation in different distant exons may also give origin to CRMpos forms (Molise and prothrombin Tokushima).

D. Clinical picture

True deficiency of the homozygote levels is always characterized by several and sometimes by severe bleeding manifestations. Intracerebral bleeding and hematomas have been reported. Table shows the bleeding symptoms presented by the patients reported in the literature. Cerebrovascular bleeding seems particularly life threatening.

Altogether, there is no possibility to reach a diagnosis of prothrombin defects on clinical grounds; the bleeding manifestations are varied, none is

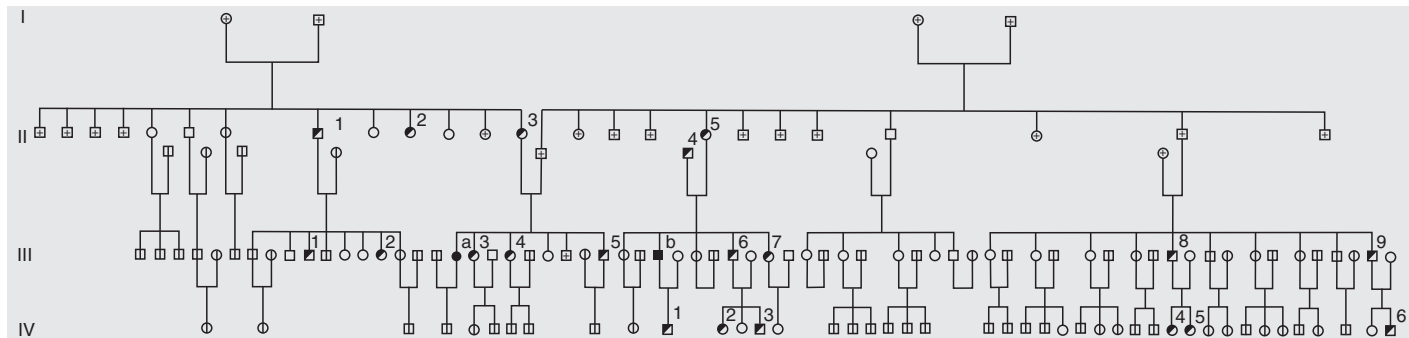


Figure 14.1 Large kindred with congenital hypoprothrombinemia (“true” prothrombin deficiency). The hereditary transmission is autosomal recessive. The same is true for congenital dysprothrombinemias. Full-darkened symbols = homozygotes (IIIa, female and IIIb male); half-darkened symbols = heterozygotes; ○ or □ died, ○ or □ alive, not studied. The two homozygotes were first cousins. The son of homozygote IIIa was subsequently found, as expected, to be heterozygote.

Table 14.5 Molecular defects seen in cases of true deficiency so far studied

References	Genotype	Mutation (exon)
Tamary <i>et al.</i> (1997)	Comp. Het. (hypo/ hypo)	Del 249/250 + Arg34Trp (8, 10)
Poort <i>et al.</i> (1997)	Comp. Het. (hypo/ hypo)	Cys138Tyr + Trp357Cys (6, 10)
Poort <i>et al.</i> (1994) and Sun <i>et al.</i> (1999)	Hom.	Tys-44 Cys (3)
Akhavan <i>et al.</i> (2000)		
Case A	Hom.	Arg -1 Gln (2)
Case B, C1, C2	Hom.	Arg -2 Trp (2)
Case D	Comp. Hom.	Asp118Tyr; Arg220Cys (6, 7)
Case E, F	Hom.	Del Lys 301/302 (9)
Case H	Hom.	Arg538Lys (14)
Case J1, J2-K	Hom.	Ser394Arg (10)
Lefkowitz <i>et al.</i> (2003)		
Kindred 1	Comp. Het. (hypo/ hypo)	Arg457Gln + exon 5/6 splice junction
Kindred 2	Comp. Het. (hypo/ hypo)	Arg457Glu deletion + 5 base pair deletion (13)
Wang <i>et al.</i> (2004)	Hom.	Glu 29 Gly (2)

The list may be incomplete.

Eponyms occasionally attributed to cases of "true" hypoprothrombinemia have been omitted. It is evident that mutations in different exons may cause similar CRM negative forms.

Letters indicate kindreds as reported by Authors. CRM, cross-reacting material; Het., heterozygotes; Hom., homozygotes; Comp. Het., compound heterozygotes.

typical and the picture may be similar to that seen in other clotting factor deficiencies ([Table 14.7](#)).

Heterozygotes are usually asymptomatic but, occasional excessive bleeding after tooth extractions, tonsillectomy or after surgical procedures have been observed ([Table 14.7](#)). In this regard, it has to be remembered that patients heterozygous for Factor VII deficiency are instead always asymptomatic.

Bleeding tendency in dysprothrombinemias is much more variable and usually less severe than in true deficiency. Altogether, there is a fair correlation between prothrombin activity and the clinical picture.

At least two prothrombin abnormalities (prothrombin Cardeza and prothrombin Salakta–Frankfurt) do not seem to be associated with bleeding.

Table 14.6 Molecular defects seen in congenital dysprothrombinemias so far investigated

Variant	Genotype	Molecular defect (exon)
Barcelona (Madrid/ Obihiro)	Hom. and Het.	Arg271Cys (8)
Padua (Dhahran)	Hom. and Het.	Arg271His (8)
Molise (Tokushima)	Comp. Het. (hypo-dys)	Arg418Trp + stop codon 174 (11, 7)
Quick I (Corpus Christi)	Comp. Het. (dys-dys)	Arg382Cys (9)
Quick II	Comp. Het. (dys-dys)	Cly558Val (12)
Salakta (Frankfurt)	Hom. and Het.	Glu acid 446Ala (12)
Himi I	Comp. Het. (dys-dys)	Met337Thr (9?)
Himi II	Comp. Het. (dys-dys)	Arg388His (9?)
Prothrombin 3 Camberra	Het.	Glu acid 157Lys (3)
Bangladesh	Comp. Het. (hypo-dys)	Arg271His + Hip518Arg (8, 14)
Nijmegen	Hom. and Het.	Glu7Lys (2)
Greenville	Het.	Arg517Glu (13)
Segovia	Hom. and Het.	Glu319Arg (9)
Perija	Hom. and Het.	Glu548Ala (14)
San Antonio	Het.	Arg320His (9)
Denver	Comp. Het. (hypo-dys)	Glu300Lys + Glu309Lys (9)
Scranton	Het.	Lys556Thr (14)
No eponym (Akhawan <i>et al.</i>)		
G	Hom.	Gly 330 Ser (9)
I	Hom.	Arg 382 His (10)
Puerto Rico 1	Comp. Het.	Arg457Gly + Glu16Gly (12;2)
Puerto Rico 2	Hom.	Arg457Glu (12)

The list may be incomplete. In this case too, the mutations claimed to be responsible for the CRM positive forms, involve several exons.

Letters indicate kindred as reported by authors. CRM, cross-reacting material; Het., heterozygotes; Hom., homozygotes; Comp. Het., compound heterozygotes.

Table 14.7 Hemorrhagic manifestations observed in 26 patients with congenital hypoprothrombinemia as reported in the literature

Hemorrhagic manifestation	No. of patients	No. of positive patients	%
Umbilical cord hemorrhages	26	4	15.4
Epistaxis	26	14	53.8
Hematomas and ecchymosis	26	17	65.3
Gingival bleeding	26	3	11.5
Bleeding after tooth extraction	26	9	34.6
Gastrointestinal or abdominal hemorrhages	26	3	11.5
Hemarthroses	26	11	42.3
Hematuria	26	2	7.6
Menorrhagias, metrorrhagias	3 ^a	3	100.0
Post-partum hemorrhages	2	2	100.0
Cerebral or intracranial hemorrhages	26	3	11.5

^a Post-puberal women.

E. Diagnosis

A correct diagnosis of prothrombin deficiency can be reached only by laboratory investigation.

Routine laboratory tests such as PT and PTT are usually slightly or variably prolonged. Note that, contrary to what is observed for defect of Factors V, VII, or X, these tests may be normal or only borderline in isolated prothrombin deficiency. This may explain, at least in part, the rarity of the defect, since many cases have probably gone undetected because routine tests were so minimally altered that no further study was carried out.

Prothrombin-time-derived tests, such as Thrombotest, Normotest, or PP test, are usually more prolonged. Once a suspicion is raised, a specific prothrombin assay has to be carried out. Several tests are available (Denson *et al.*, 1971; Hjort *et al.*, 1955). The one-stage standard assay using tissue thromboplastin as activating agent and a prothrombin-free substrate is the most widely used. Provided the substrate is adequate, the test is reliable and sufficient for screening purposes (Girolami *et al.*, 1974b). The two-stage method is rarely used at present (Ware and Seegers, 1949).

Homozygous patients with “true” deficiency have usually prothrombin activity and antigen levels of less than or about 10% of normal, regardless of the method used (Table 14.8). Complete absence of prothrombin seems incompatible with life. Patients heterozygous for the same defect usually have prothrombin levels between 40% and 60% of normal. On repeated assays, a good separation of heterozygotes from normal, unaffected members

Table 14.8 Prothrombin level (%) in three homozygous patients with true hypoprothrombinemia seen in Padua as determined by different techniques

Method	Patient 1	Patient 2	Patient 3
Classical one-stage method (Quick, 1946)	15	9	16
Iowa two-stage method (Ware and Seegers, 1949)	13	11	12
Notechis scutatus scutatus viper venom (Jobin and Esnouf, 1966)	14	12	16
Taipan viper venom (Denson <i>et al.</i> , 1971)	12	10	11
Echis carinatus venom (Girolami <i>et al.</i> , 1976)	10	11	12
Chromogenic (S2238)	13.6	14.6	9
Staphylocoagulase (Soulier and Prou-Wartelle, 1966)	9	10.5	11
Immunological (Mancini <i>et al.</i> , 1965)	12.5	11.5	11.5

of the family may be achieved. In a group of 26 heterozygotes, we found an average of 51% (range 43–75). These values were easily distinguishable from a group of normal relatives (mean 98; range 84–130).

Other tests, using other activating substances, are needed to screen for potential abnormalities. Regardless of the assay used, the activity level in case of “true” deficiencies should be about the same. Should one encounter a discrepancy in the activity level, a dysprothrombinemia should be suspected. In this regard, the Echis Carinatus venom assay may be important, since it has been reported to be normal in a few dysprothrombinemias (Girolami *et al.*, 1984). It has to be remembered that prothrombin-activating agents used in clotting tests have different methods of converting prothrombin into thrombin. This is particularly true for viper venoms. The four major venoms used in prothrombin assays have, in fact, different requirements. Tiger snake venom behaves as a tissue thromboplastin requiring phospholipids, Factor V, and calcium. On the other hand, Echis Carinatus venom converts prothrombin directly without depending on other factors. An intermediate position is shown by Taipan viper venom and Australian brown viper venom which do not require Factor V, but do require phospholipids and calcium for optimal activity (Table 14.9) (Denson *et al.*, 1971; Masci *et al.*, 1988).

Chromogenic substrates have been used for prothrombin assay (Girolami *et al.*, 1980b). In this regard, it has to be kept in mind that clotting activity is not always equivalent to chromogenic activity. In fact, chromogenic prothrombin assay has been normal in prothrombin Padua and higher than expected in prothrombin Molise.

Table 14.9 The variable requirements of several one-stage Factor II assays

Assay	Factor V	Phospholipid	Ca ²⁺	Activating agents
Classical one stage	+	+	+	Tissue thromboplastin
Tiger snake venom	+	+	+	Tiger snake venom
Taipan viper venom	No	+	+	Taipan viper venom
Textarin time	No	+	+	Pseudonaja textiles venom
Echis Carinatus venom	No	No	No	Echis Canaritus venom
Staphylocoagulase	No	No	No	Staphylocoagulase

An immunological investigation is essential (electroimmunoassay, ELISA, radial-immunodiffusion, laser nephelometer, etc.) (Girolami *et al.*, 1982b; Mancini *et al.*, 1965). Without a careful antigen evaluation, patients with prothrombin deficiency cannot be adequately classified. Even molecular biology studies are unable to substitute for an antigens assay, since no specific group of mutations has been associated with either surely CRMpos or CRMneg forms.

The staphylocoagulase is a clotting method that employs as activating agent staphylocoagulase, derived from certain strains of *Staphylococcus aureus* cultures. It complexes with prothrombin to form thrombin coagulase that, in the presence of an excess of fibrinogen, yields a clotting time that depends only on the level of prothrombin antigen (Soulier and Prou-Wartelle, 1966).

Difference between clotting and antigen activity <15% is probably of little significance, if any. One may still be dealing with a hypoprothrombinemia. Discrepancies higher than this support dysprothrombinemias.

If a dysprothrombinemia is suspected, further studies are indicated. Prothrombin clotting assay using different activation agents (Taipan viper venom, Echis carinatus venom, etc.) can be used. Bidimensional immunoelectrophoresis on plasma and serum to detect abnormal migration of whole prothrombin or prothrombin split products in serum is another alternative. Some dysprothrombinemias will show abnormal migration of plasma prothrombin, either less anodic or more anodic (Table 14.10). Other dysprothrombinemias show an abnormal pattern in the migration of serum prothrombin. Molecular biology techniques are absolutely indicated to further characterized the defect (Fig. 14.2). These have become almost routine when investigating new cases. Database of mutations is being continuously enriched. Unfortunately, the genotype–phenotype relation

Table 14.10 Plasma (or serum) prothrombin immunoelectrophoretic patterns of some abnormal prothrombins

Normal	Less anodic (slow)	More anodic (fast)
Padua (serum abn.)	Prothrombin 3 (Canberra)	Barcelona
Molise (serum abn.)	Segovia	San Juan (serum abn.)
Cardeza (serum abn.)	Denver	Habana (serum abn.)
Clamart	—	—
Houston	—	—

Abn. = abnormal.

is not completely clear yet (Akhavan *et al.*, 2003). Recently, three-dimensional putative models have been constructed (Akhavan *et al.*, 2000).

F. Prognosis

Prognosis is guarded for homozygotes and good for heterozygotes. Because of the frequency of cerebrovascular bleeding in homozygote patients, morbidity and mortality are high. The eldest alive patient is a 61-year-old female (Girolami, 1971). In the case of dysprothrombinemias, prognosis seems better. In fact, the eldest patient described is a 75-year-old man (Weinger *et al.*, 1980). There are few studies available concerning the effect of a prophylactic approach in these patients. The long survival time of infused prothrombin could justify this approach (Biggs and Denson, 1963).

G. Therapy

Substitution therapy for prothrombin deficiency is carried out by administering plasma, prothrombin concentrates, or both. Only homozygotes need prompt treatment in case of bleeding or adequate prophylaxis before surgical procedures. Since no isolated Factor II concentrate exists, concentrates of PC factors are used (Lechler, 1999). The problem with these concentrates results from the fact that they are often titrated in Factor IX content and not in Factor II. This may cause some discrepancy between the amount (units of Factor IX) administered and the *in vivo* recovery (Factor II level). More recent Factor IX concentrates, monoclonal or recombinant, should be used since these allegedly do not contain any Factor II. The long half-life of infused prothrombin (about 70 h) allows infusion every 2–3 days with a satisfactory yield (Biggs and Denson, 1963; Soulier *et al.*, 1962). Oral or intravenous antifibrinolytic agents have also been used. Because of the rarity of the condition, there are no reports on liver transplants or gene therapy attempts. The oral contraceptives have been shown to be beneficial with a sharp decrease of menometrorrhagia and improved hematocrit.

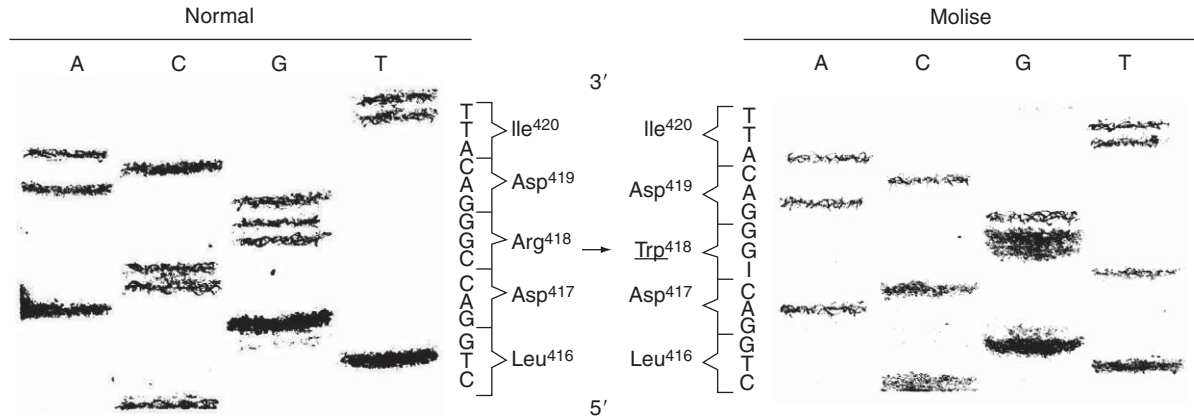


Figure 14.2 Autoradiographs showing the prothrombin Molise gene defect. A missense mutation, C to T at nucleotide position 9,490 in Exon 11, results in coding for Trp (TGG) instead of Arg (CGG) at amino acid position 418.

H. Combined defects

The most important combined defect of the prothrombin is the association with Factors VII, IX, X, and Proteins C and S due to the malfunction or absence of one of the enzymes involved in vitamin K metabolism. This combined defect will be dealt with in a separate heading. Casual associations of prothrombin with other isolated clotting factors have never been described.

III. CONGENITAL FACTOR VII DEFICIENCY

The first case of congenital deficiency of Factor VII (FVII) was described by Alexander ([Alexander *et al.*, 1951](#)), and the first review appeared in 1959 ([Dische and Benfield, 1959](#)). Since that date, many additional cases have been reported. Owen *et al.* in 1964 gathered only 60 sufficiently proven cases. In 1972, we had gathered 87 fairly proven cases. Subsequently, two other reviews were published in 1981 and 1984, which brought up-to-date information contained in the previous ones ([Girolami *et al.*, 1984](#); [Ragni *et al.*, 1981](#)). Several additional cases have been reported in the past two decades, together with molecular investigation of the defect, and the database is always expanding ([McVey *et al.*, 2001](#)). Probably, several cases went unreported, since they were deemed to add very little, if any, to the knowledge of the disease. These figures indicate that the defect is still rare but is definitely more frequent than Factor X or Factor II deficiency. Its frequency can be estimated around 1/300,000 people, definitely higher even than that for Factor V deficiency.

Recent reviews appeared in 1995, 1998, and 2002 mainly dealt with molecular biology changes or with both congenital and acquired alterations forms ([Cooper *et al.*, 1997a](#); [Iannello *et al.*, 1998](#); [Perry, 2002](#); [Tuddenham *et al.*, 1995](#)). Chapters dealing with FVII published in recent editions of widely used books are sometimes not completely satisfactory because information is scattered in different chapters.

The purpose of this chapter is to combine clinical, genetic, laboratory, and molecular biology data dealing with FVII deficiency and abnormalities in a unified presentation.

A. Classification and prevalence

Until a few years ago, it was thought that Factor VII deficiency represented a single entity in which a defective Factor VII synthesis occurred. The first demonstration of the presence of an excess of CRM over the clotting counterparts was given by [Goodnight *et al.* \(1971\)](#). Since then other reports

have confirmed the findings. In some instances, the FVII protein level was reported normal. It was then concluded that Factor VII defect could be subdivided into three major variants, as namely, Factor VIIneg (CRM negative), Factor VIIred (CRM reduced), and Factor VIIpos (CRM positive) (Cooper *et al.*, 1997a; Goodnight *et al.*, 1971; Mazzucconi *et al.*, 1977). These findings are in agreement with what demonstrated for prothrombin, for Factor IX and Factor X.

A classification based only on the level of activity and antigen is untenable, since it does not take into account the different reactivity toward different tissue thromboplastins shown by some of these abnormal FVII. In fact, it was demonstrated in 1978 that an abnormal FVII, namely, a Factor VIIpos variant, may either be inert toward some tissue thromboplastin or be normally activated by others (Girolami *et al.*, 1978a, 1979; Triplett *et al.*, 1985); therefore, different levels of FVII can be obtained using different tissue thromboplastins. This discovery has radically changed the approach toward the classification of FVII defects. The correct approach is the following:

1. Factor VII “true” deficiency (homozygous and heterozygous)
2. Factor VII abnormalities (homozygous, heterozygous, and compound or double heterozygous)
 - a. Forms with discrepancy between clotting and antigen levels
 - b. Forms not only with the same discrepancy but also with different behavior toward tissue thromboplastins of different origin.
3. Combined Factor VII defects with other clotting factors

Factor VII CRMneg are those cases with no detectable or very low antigen and therefore no activity (<10%). Factor VIIred are those with reduced FVII antigen (usually 20–50% of normal). However, such levels are always definitely higher than the clotting counterparts. Finally, Factor VIIpos are those cases in which FVII activity level is always low and FVII antigen is normal. The exact collocation of Factor VIIred may be difficult, since sometimes a similar pattern (intermediate FVII antigen and low FVII activity) may be due to the presence of a compound or double heterozygosis (hypo-dys form). Eponymous should be applied only to abnormalities, CRMpos.

The exact incidence of CRMpos variants has not been surely established yet (Girolami *et al.*, 1979, 1984; Goodnight *et al.*, 1971; Mazzucconi *et al.*, 1977). The overall picture would suggest that about 50% of patients show no FVII antigen in their plasma, namely, are CRM negative. This is a little less than that described for hemophilia B where about 60% of cases are B negative. Using another approach, FVII defects with low but parallel levels of FVII activity and antigen may be termed Type I (cases of true deficiency), and those with low activity and normal or reduced antigen may be defined as Type II.

As far as mechanisms or function are concerned, FVII deficiency may be subdivided into: (1) defects in secretion, (2) defects in binding to tissue factor (TF), (3) defects in carboxylation, and (4) defects in Factor X activation.

Molecular biology studies are supplying useful information for a satisfactory classification. However, the picture is not clear yet. The same point mutation has been described in patients who showed a different clotting and antigenic pattern. On the contrary, a similar clotting and antigenic pattern has been described in patients with different mutations (Arbini *et al.*, 1994; Bernardi *et al.*, 1993, 1994; Chaing *et al.*, 1994; Fromovich-Amit *et al.*, 2004; Gamba *et al.*, 1998; Gomez *et al.*, 2004; James *et al.*, 1993a; James *et al.*, 1993b; James *et al.*, 1997; Kuppaswamy *et al.*, 1993; Mariani *et al.*, 2005; Peyvandi *et al.*, 2000; Shurafa *et al.*, personal communication; Wu *et al.*, 2006; Wulff and Herrmann, 2000).

B. Biochemistry

Factor VII is one of the PC factors. It is synthesized in the liver in the presence of vitamin K together with Factors II, IX, and X and Protein C and Protein S.

It is a single polypeptide glycoprotein of a molecular weight of about 50,000 Da.

Like the other PC factors, Factor VII has in its COOH-terminal region a number of glutamic acid residues that are, in the presence of vitamin K, converted to γ -carboxyglutamic residues. The plasma concentration of FVII is about 0.1 mg%, namely, about 1/10 the concentration of Factor X and about 1/20 the concentration of prothrombin. This very low concentration explains why FVII could not be easily demonstrated by precipitating techniques such as by electroimmunoassay, immunodiffusion, or radial immunodiffusion. It has a short half-life of about 4–6 h.

Although FVII appears to be an active enzyme, its activity is increased about 40-fold when converted to its active form by Factors Xa, IXa, and XIIa or through Factor VIIa. Factor VIIa is a two-chain serine protease of 26,000 and 22,000 Da, held together by disulfide bonds.

The activation of factor VII occurs after binding to TF by cleavage of a single bond (Arg152–Ile153). Activated FVII requires TF and calcium to show significant proteolytic activity toward its substrates, namely, Factors IX and X.

FVII, similarly to other vitamin K-dependent clotting factors, contains several domains, namely, a Gla domain, epidermal growth factor (EGF)-like or kringle domains and a C-terminal catalytic domain homologous to trypsin.

FVIIa by itself has only a weak enzymatic activity and has a half-life similar or even shorter than that of the zymogen.

FVII seems to be the only known ligand to TF, and the complex formation is regulated by a specific inhibitor, the TF pathway inhibitor (TFPI),

which forms a quaternary complex involving FVII and both the TF–FVIIa complex and Factor X. The role of TFPI in Factor VII deficiencies has not been extensively studied yet.

The heavy chain of FVIIa contains the catalytic domain, whereas the light chain of FVIIa contains the γ -carboxyglutamic acid (Gla) residues involved in calcium and membrane phospholipid binding. FVIIa is normally present in the circulation at trace levels, and it is maintained to serve as the physiological initiator of coagulation.

C. Genetics and hereditary transmission

The hereditary transmission of Factor VII deficiency is autosomal recessive or incompletely recessive (Alexander *et al.*, 1951; Cooper *et al.*, 1997a; Girolami *et al.*, 1984). Consanguinity is present in about 30% of families. The FVII level usually found in homozygous patients with cause deficiencies is below 10% of normal. By definition, all these patients have also low or undetectable levels of FVII antigen. This hereditary pattern seems to be present even in the case of abnormalities (Factor VII Padua, for example). In this regard, it is important to remember that, as seen for other PC factors, an abnormality may be associated with a “true” deficiency or with a different abnormality of the same factor (double or compound heterozygotes).

Such combined or double heterozygotes may represent a difficult diagnostic problem, and the evaluation of the antigen level is crucial in the understanding of the hereditary pattern. Parents or children of homozygous patients are obligatory heterozygous. The FVII level usually found in these heterozygous patients varies from 40% to 60% of normal.

Molecular biology analysis may be necessary to clarify the hereditary pattern in some families. However, because of their technical difficulties these studies have no widespread use in routine clinical and laboratory practice.

The human Factor VII gene is located on the long arm of chromosome 13 (q34), near the Factor X gene. It is composed of 9 exons and 8 introns with an overall organization similar to Factor IX, Factor X, and Protein C.

Exons 1 and 2 seem involved in a single sequence; Exon 2 encodes the propeptide and the Gln domain; Exon 3 is responsible for a hydrophobic region; Exons 4 and 5 control the EGF domains; Exons 6 and 7 control the activation process; and, finally, Exon 8 encodes the catalytic domain (Tables 14.11 and 14.12).

At present, there are about 300 patients who have been investigated from a molecular point of view. Several of these mutations are identical, even though sometimes the phenotype appears different. For example, there are at least five families with FVII deficiency (Padua, Little Rock, Detroit, Richmond, and Verona) due to the same mutation, namely, the Arg304Gln in Exon 8. This may create confusion and should be avoided. The first family described should be kept as the index case. Minor laboratory

Table 14.11 Selected and representative examples of kindreds with true deficiency (Type I)

References	Activity	Antigen	Clinical picture	Molecular defect	Functional defect	Genotype
Tuddenham <i>et al.</i> (1995)	1.7	1.2	Severe	Del C10696 Del C10785	Catalytic	Comp. Het.
Arbini <i>et al.</i> (1994)	<2	<4	Moderate	Ala294Val Del C11125	Catalytic	Comp. Het.
Peyvandi <i>et al.</i> (2000); (Kindred B1)	<1	10	Severe	Gln100Arg	EGF domain	Hom.
Wulff and Herrmann (2000)	8	4	?	G64A	Pro-peptide	Hom.
Fromovich-Amit <i>et al.</i> (2004)	5.5	6	Mild	Ala244Val	Catalytic	Comp. Het.
Gomez <i>et al.</i> (2004)	0.4	1	Severe	Gly117Arg Arg152 X	Secretion Stability	Comp. Het.

Comp. Het., compound heterozygotes; Hom., homozygotes.

Table 14.12 Selected and representative examples of genetic variants of Factor VII defects with normal or near normal antigen (CRM positive or Type II forms)

Variant	Activity (% of normal)	Antigen	Clinical picture	Molecular defect	Functional defect	Genotype
Kansas (Kuppuswamy <i>et al.</i> , 1993)	4	60%	Asymptomatic	Gln100Arg	Defective binding	Comp. Het.
Padua (James <i>et al.</i> , 1993b)	9 (*)	100%	Moderate	Arg304Gln	Impaired activation	Hom.
Charlotte (Chaing <i>et al.</i> (1994)	<1	100%	Severe	Arg152Gln + Arg79Gln	Decreased binding to TF + impaired activation	Double Hom.
Kindred H (Peyvandi <i>et al.</i> (2000)	<1	135%	Severe	Pro303Thr	Catalytic domain	Hom.
No eponym (Fromovich-Amit <i>et al.</i> (2004))	<1	83%	Mild	Cys310Phe	Catalytic domain	Hom.

Only homozygotes or compound heterozygotes have been taken into consideration.

* Normal with ox-brain thromboplastin; Comp. Het., compound heterozygotes; Hom., homozygotes.

Table 14.13 Different behavior in clotting and immunological assays of Factor VII abnormalities due to the same amino acid substitution (Arg304Gln)

Defect	Factor VII activity (% of normal)			Factor VII antigen (% of normal)
	Rabbit-brain	Human brain or placenta	Ox-brain	
FVII Padua	8	30	100	100
FVII Verona	20	20	50	60
FVII Little Rock	7	16	30	67
FVII Detroit (*)	14	21	124	80
FVII Richmond	n.r.	14	n.r.	120

Differences may be explained either by different procedures or by the presence of additional, unknown defects.

* Pattern is more close to that observed in FVII Padua; n.r., not reported.

differences seen in these patients may be either due to the different laboratory techniques or due to the presence of other unknown molecular changes (Table 14.13). The same mutation was first seen in an isolated patient by O'Brien *et al.*, but no family study or extensive clotting investigation was supplied (O'Brien *et al.*, 1991).

By eliminating the duplets, one reaches the approximate number of about 200. These unique alterations include missense mutations (the large majority), splice junction and promoter changes (rare) and nonsense mutation, deletion or insertions (very rare). All areas of the gene are involved (promoter, Gla domain, etc.). The large majority involve Exon 8. Several areas or domains seem involved in the binding to TFs, and this may explain the different results observed sometimes using thromboplastins of different origin. As far as FVII activation is concerned, residues between 300 and 330 seem critical. Several substitutions in this area of Exon 8 have been associated with low activity and normal antigen (CRM positive). Furthermore, peptide inhibition studies have shown that residues 330–340 are important for both Factor IX and Factor X binding and activation (James *et al.*, 1997).

On the contrary, substitutions on Exons 1–7 are more frequently associated with cases of true deficiency (CRMneg). Recent studies have indicated that the Cys329 residue plays a critical role in FVII function (Wu *et al.*, 2006).

A large number of molecular studies have not been as rewarding as originally expected. It is becoming every day more evident that different mutations may give the same or similar phenotype and, conversely, that different phenotypes may be caused by the same mutation (ex Arg304Gln).

From a clinical point of view, a complete and accurate evaluation of FVII levels activity and antigen appears sufficient.

D. Clinical picture

There is no typical clinical feature for Factor VII defect. Only homozygotes are variably symptomatic. Heterozygotes are always asymptomatic contrary to what is showed for heterozygotes with Factor II and Factor X deficiency (Table 14.14). The wide clinical spectrum of hemorrhagic manifestations justified the fact that in some cases a minor bleeding is maintained to be present; in others, a major hemorrhagic condition is described. Such variability seems to correlate only partially with FVII levels, which in homozygous patient is usually below 10% of normal (Giansily-Blaizot and Schved, 2005). The discrepancy seen in some patients between FVII levels and bleeding tendency remains to be clarified. It has to be observed that this discrepancy may sometimes be more apparent than real because of the different levels of FVII activity obtained using tissue thromboplastins of different origin. If comparison is not made with reagents that yields the lowest FVII activity level, wrong conclusions could be drawn. Bleeding tendency is characterized by easy bruising, gastrointestinal bleeding, hematuria, epistaxis, and, in several cases, hemarthrosis, occasionally with severe orthopedic impairment. Menometrorrhagias may be present and severe in women. Oral contraceptives have been shown to decrease this type of bleeding. An increased incidence of cerebrovascular bleeding has also been demonstrated. This is often the cause of death or serious neurological sequelae. As said before, there is nothing typical in this bleeding tendency. Note that spontaneous or, more frequently, replacement therapy-associated thromboembolic manifestations have been described in FVII deficiency, indicating that a FVII defect is unable to fully protect from thromboembolic phenomena (Girolami *et al.*, 1978a; Sabharwal *et al.*, 1992). Pregnancy has been brought to term under adequate substitution therapy.

E. Laboratory diagnosis

The main criteria for the diagnosis of Factor VII deficiency are: prolonged prothrombin time corrected by the addition of normal plasma or serum but not by the addition of adsorbed plasma, and normal partial thromboplastin and thrombin time. In the past, it was maintained that a prolonged prothrombin time together with a normal Roussel viper venom (RVV) clotting time was sufficient for a sure diagnosis. This has been demonstrated not to be the case, since a Factor X abnormality, Factor X Friuli, also shows a similar pattern (Table 14.15). In the latter case, however, a prolonged PTT is always present. Furthermore, there are rare families with Factor X deficiencies, which appear defective only in the extrinsic system. As a consequence, the pattern obtained is identical to that seen in FVII deficiency, namely, normal PTT, prolonged PT, and normal TT.

Table 14.14 Comparison in incidence of minor bleeding manifestations between heterozygotes for Factor II or Factor VII or Factor X deficiency and hemophilia B carriers and unaffected family members as seen in Padua

Defect	No. affected	Symptomatic No. (%)	No. of nonaffected	Symptomatic No. (%)	Comments
Factor II	26	9 (34.6)	31	1 (3.2)	Epistaxis, easy bruising, menorrhagia, excessive bleeding after tooth extraction or surgery
Factor VII	60	2 (2.4)	52	2 (3.8)	
Factor IX (hemophilia carrier)	38	1 (2.6)	45	2 (4.4)	
Factor X	120	36 (30)	81	2 (2.4)	

The difference is significant only for Factors II and X defects.

Table 14.15 Laboratory differential diagnosis between Factors VII and X deficiencies and some variants

Defect	PTT	PT rabbit- brain/ ox-brain	RVV	Antigen (CRM)	Comments
Factor VII true deficiency	N	P/P	N	No	
Factor VII Variant VII Padua	N	P/N	N	Yes	Other variants may have different pattern
Factor X true deficiency	P	P/P	P	No	
Factor X Variant X-Friuli	P	P/P	N	Yes	Other variants may have different pattern
Factor X Variant X-Padua	N	P/P	N	Yes	Other variants may have different pattern

CRM, cross-reacting material; RVV, Roussell viper venom; P, prolonged; N, normal.

Prothrombin time derivate tests, such as PP test, Normotest, and Thrombotest, are all variably prolonged. FVII activity is low, as expected. FVII activity levels are usually below 10% of normal in homozygote patients and between 40% and 60% of normal in the heterozygote population.

More variable levels may be encountered in compound heterozygotes, especially in dys-dys forms. Therefore, a sure allocation of heterozygotes may be complicate in the case of compound or double heterozygosity. In this case, the independent segregation of the two defects should be encountered in the family members. Factor VII assays can be carried out as follows: (1) using a tissue thromboplastin as activator in a one-stage procedure, (2) by means of chromogenic substrates, (3) by neutralization test using anti-FVII antiserum, (4) by an ELISA method, and (5) by radioimmunoassay.

Only the first one is widely used. It is important to use at least two tissue thromboplastins (e.g., rabbit-brain and ox-brain thromboplastins). If no major discrepancy is present in the FVII levels so obtained, the defect is probably a "true" FVII deficiency. If a discrepancy over 15% in the FVII level is present between the two clotting assays, a FVII abnormality should be suspected. In this case, thromboplastins of other origin, human for example, should also be used. The substrate best suited for such assay is represented by fresh or lyophilized congenital FVII deficient plasma.

If lyophilized or stored plasma is used, a mixture of such plasma with absorbed normal plasma is preferable to assure adequate Factor V levels in the system. Artificially prepared substrates are not satisfactory. The routine use of chromogenic assays is not practical so far, since the method requires the presence of purified Factor X or a three-stage rather complicated assay (Margaritella and Deutsch, 1981; Seligsohn *et al.*, 1978). Furthermore, it is more complicated and expensive than the plain clotting test. In the characterization of an abnormality, it is imperative to carry out also an immunological assay. The neutralization test was widely used in the past often with equivocal or doubtful results.

The neutralization test is based on adding antiserum to the test plasma (first mixture or stage I). Subsequently, after 1 h incubation at 37 °C, an aliquot is mixed with equal parts of pooled normal plasma (second mixture or stage II). Finally, FVII is assayed in this second mixture. The residual FVII activity found in the second mixture is inversely related to the presence of FVII protein in the test plasma. In the evaluation of FVII abnormalities, it is important to use in the assay of residual FVII activity (stage III), the thromboplastin that yields the lowest activity (Girolami *et al.*, 1984). The radioimmunoassay is surely more precise but has the limitation of the need of I¹²⁵ labeled FVII. The availability of suited precipitating antisera has simplified the matter allowing the performance of, for example, an electroimmunoassay or a construction of an ELISA. The ELISA method has also been introduced in recent years and has acquired widespread use. It seems superior and less time consuming than the neutralization test and the radioimmunoassay (Fair, 1983).

The correct identification of the heterozygotes is usually fairly simple, but in some patients with borderline FVII activity values (60–70% of normal) the procedure may be difficult. In our hands, the best approach to that has been the accurate and repeated FVII activity assays on different occasions. In heterozygotes, the level will be found constantly even though only slightly decreased. Patients with acquired conditions tend to present variable levels. Furthermore, the administration of vitamin K will usually correct the defect only in acquired conditions. Finally, in acquired defects there is always a concomitant decrease in other factors, usually Factors II, IX, and X.

The evaluation of activated Factor VII (FVIIa) has been proposed, but its value, in clinical practice, remains to be proven (Morrissey *et al.*, 1993). The principle of the test is based on the discrepant sensitivity of a recombinant soluble TF toward FVII and FVIIa. This particular TF (truncated TF) does not activate FVII into FVIIa and therefore is maintained to be independent of the FVII present in the plasma. In other words, it measures only FVIIa already existing in the plasma. Normally about 99% of FVII circulating in plasma is the zymogen form and about 1% circulates in the active form. Molecular biology techniques, inclusive occasionally of expression studies,

have been carried out in the most recent era. This has greatly contributed to the characterization of several variants (Fig. 14.3).

Of paramount importance in the diagnosis of congenital FVII defects, regardless of the type, are the following criteria: (1) fixity of the defect; (2) no influence by vitamin K administration but for the case of combined Factors II, VII, IX, and X; and (3) the presence of similar defects in the family.

F. Prognosis

The prognosis of congenital FVII deficiency is guarded. The life expectancy varies considerably and is in a fair relationship with the FVII activity level. The improved therapeutic approach due to the availability of FVII concentrates is having a positive effect on survival. However, no study is available on the survival of these patients yet.

Cerebral hemorrhages seem a frequent cause of death in patients with very low-activity levels (Ragni *et al.*, 1981). On the contrary, other patients with FVII activity of about 10% of normal lead an almost normal life (...). Heterozygous have an excellent prognosis, since they are always asymptomatic.

G. Therapy

Factor VII has a short survival half-life, about 5 h, and therefore substitution therapy may be a problem in the presence of a severe bleeding emergency (traumas, surgery, cerebrovascular bleeding, etc.). The administration of fresh frozen plasma may not be practical because of the low yield. Prothrombin complex concentrates, titrated for Factor IX, contain variable amounts of FVII. In our hands, the use of such concentrates resulted in satisfactory FVII levels (Fig. 14.4), but it entailed in the past a very high incidence of nonA-nonB hepatitis. Approximately 1 of 2 patients developed clinical and/or laboratory evidence of hepatitis. The pattern has improved in recent years with better sterilization procedures. Safe therapeutic levels of FVII seem to be around 30–40% of normal. This is in agreement with the known fact that heterozygotes, which have about 50% activity levels, do not show bleeding manifestations. Isolated Factor VII concentrates, recombinant or not, are also available. The effect of antifibrinolytic agents on survival times has not been investigated. On the basis of the experience gathered from other clotting factors, it could be claimed that the association of such compounds together with substitution therapy may be beneficial.

During the past decade, a concentrate of recombinant-activated FVII has been widely used in the treatment of Factor VII deficiency (Brenner and Wiis, 2007). We think this is not always justified both for reasons of costs and for the danger of thrombotic complication which, even though rarely, do occur (O'Connell *et al.*, 2006). Furthermore, there is no

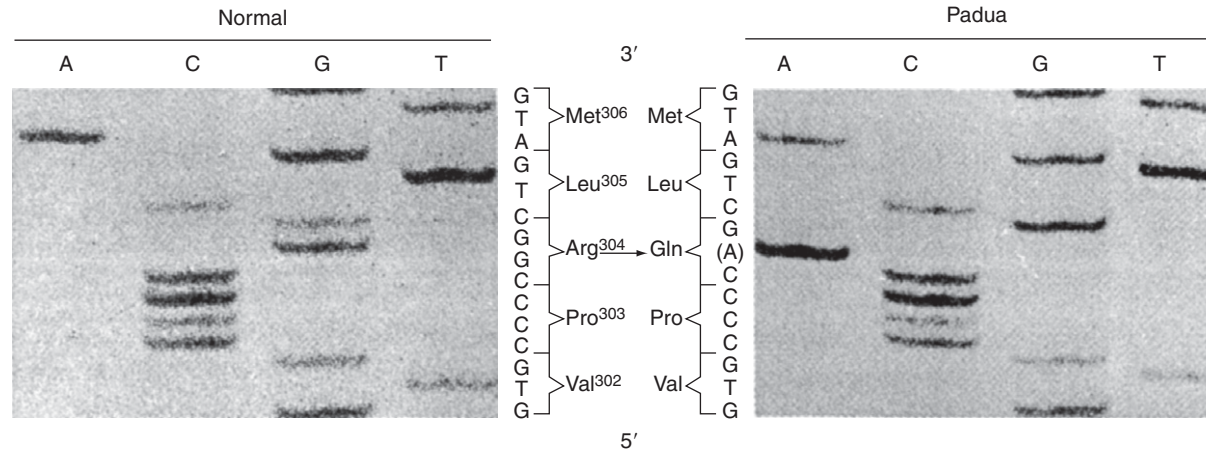


Figure 14.3 Autoradiography showing the point mutation at residue 10,828 in Exon VIII of FVII Padua gene. The base substitution is shown in parentheses. The mutation results in coding for glutamine instead of arginine at position 304 of the abnormal FVII molecule.

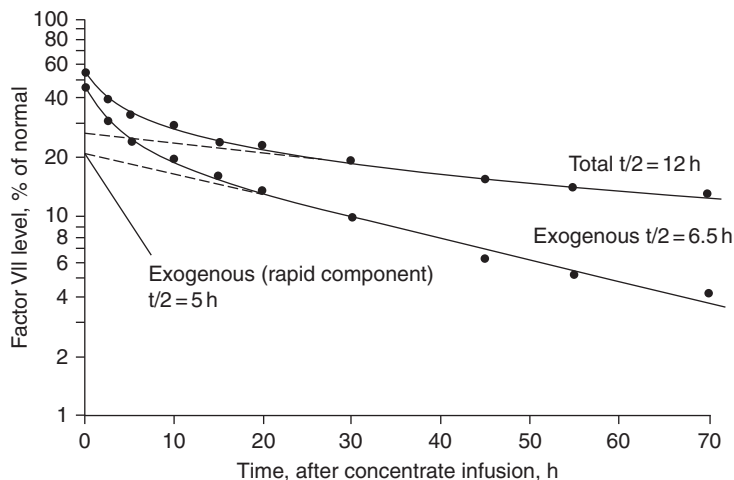


Figure 14.4 This FVII deficient patient received 1,000 units of Factor VII concentrate together with 300 ml of fresh plasma (about 15 units/kg body weight) in 30 min. The survival time of exogenous component was 6.5 h.

available demonstration of the superiority of this concentrate with regard to the standard FVII concentrates. Oral contraceptives have been shown to decreased menorrhagia and improved anemia and also to prevent ovulation-induced hemoperitoneum (Girolami *et al.*, 2006; Payne *et al.*, 2007). For the sake of completion, it has to be remembered that orthotopic liver transplant is a potential therapeutic option. It has been reported so far in four cases with apparent good results (Dhawan *et al.*, 2004; Levi *et al.*, 2001). Two of such patients, before transplantation, had undergone injection of hepatocytes in the portal system with only temporary and partial increase of FVII level (Dhawan *et al.*, 2004). On the contrary, no data are available with regard to attempts of gene therapy.

H. Association with other clotting and non-clotting defects

In addition to the bleeding condition due to the combined defect of all vitamin K-dependent clotting factors, which will be dealt with separately, occasional reports of the association of FVII with other clotting factors have been reported in a given patient and in his or her family.

These associations are important because they could create diagnostic difficulties.

These combined defects may be subdivided for simplicity in: (a) association with one of the other PC factors, (b) association with non-vitamin K-dependent factors, and (c) association with non-clotting abnormalities.

1. Association with other prothrombin complex factors

Several reports have dealt with families in which the concomitant presence of two of these factors was present. Two single PC factors have been reported in association with FVII deficiency, namely, Factor IX and Factor X.

The association with Factor IX, combined defect FVII + FIX, has received considerable attention in the past. However, the condition has never been fully understood and accepted.

In some families, there seem to be an independent segregation of the two defects, namely, hemophilia B and heterozygous FVII deficiency (Hall *et al.*, 1975). In some instances, the combined defect has been maintained to be due to a laboratory artifact (low FVII level in hemophilia B plasmas, especially hemophilia BM plasmas).

Unfortunately, no molecular studies in these patients have been carried out, and therefore no final conclusion can be drawn as to the significance of this combined defect.

The second condition, namely, combined FVII and Factor X deficiency, apparently consists of two forms. In the first form (Type I), there is a casual association of the two defects in the same family. At least, few families have been described. In these families, there is an independent segregation of the two defects (Boxus *et al.*, 1997; Kroll *et al.*, 1964; Menegatti *et al.*, 2004).

However, another form of combined FVII and Factor X defect has been reported. This form is due to alteration in chromosome 13 (q.34) where both FVII and Factor X genes are located (Ott and Pfeiffer, 1984). In this case, there is no independent segregation of the two defects in the family, and the hereditary pattern is autosomal dominant (Ott and Pfeiffer, 1984; Pfeiffer *et al.*, 1982).

Often, these patients have also malformations and defects unrelated to blood coagulation such as mental retardation and bone abnormalities.

2. Association with non-vitamin K-dependent factors

Other associated coagulative defects have been reported with Factor VIII or Factor V deficiencies (Ambriz-Fernandez *et al.*, 1985; Gaston *et al.*, 1961; Girolami *et al.*, 1977b, 2007; Machin and Miller, 1980).

The significance of these combined defects remains to be clarified. It seems that two types of combined Factors VII and VIII exist (Girolami *et al.*, 1977b). The first type consists in the chance association of heterozygous Factor VII deficiency with hemophilia A. The second type of combined defects is more interesting in the sense that it seems to be secondary to a common alteration involving both factors. However, this has not been proven.

A few families with combined Factor VII and Factor V defects have been studied (Ambriz-Fernandez *et al.*, 1985; Girolami *et al.*, 2007; Rotoli *et al.*, 1983). One of these cases has been investigated by molecular biology

techniques and confirmed to be due to casual association between defects independently derived from parents (Traynis *et al.*, 2006).

Occasional association with von Willebrand disease has also been reported. In this case, an independent segregation of the two defects noted in the families has been demonstrated (Girolami *et al.*, 2007). Other associations were described with Factor XI and Factor XII (Sleese and Schumacher, 1977; Berube *et al.*, 1992).

Because of the relative diffusion of the defect, it is of no surprise that these combined defects have been described. Even an association with Protein C deficiency has been described (Takeuchi *et al.*, 1988). Interestingly, this patient had pulmonary embolism.

3. Association with non-coagulative abnormalities

The other diseases associated with FVII defect were some bilirubin metabolism diseases such as Gilbert or Dubin–Johnson (D–J) (Seligsohn *et al.*, 1969, 1970a). These defects, particularly in D–J, appear frequently in certain Jews populations, and the association with FVII defect may represent the result of an independent hereditary transmission (Seligsohn *et al.*, 1970b). Recent investigations have shown that one of the mutations involving the multi-organ resistance Protein Z transporter causing D–J, namely, the I1173F, was present in Iranian Jewish, whereas another, the R1150H, was present in Moroccan Jews. Since both mutations were found associated with FVII deficiency due to the same A244V missense mutation, it was concluded that the D–J mutations occurred later in time, namely, after the Jewish migration to these countries, about 600 BC (Mor-Cohen *et al.*, 2007).



IV. CONGENITAL FACTOR IX DEFICIENCY (HEMOPHILIA B)

The most astonishing fact about Factor IX (FIX) deficiency is its vitamin K dependency but its sex-link hereditary transmission, whereas all other vitamin K-dependent factors have an autosomal pattern of inheritance.

The isolated deficiency of FIX is termed hemophilia B.

This defect was first recognized because of the observation by Pavlovsky in the late 1940s that there was a correction of the defect when the plasma of two “hemophilic” patients was mixed (Pavlovsky, 1947).

A few years later, the distinction was clearly established by Aggeler *et al.* and Biggs *et al.* (Aggeler *et al.*, 1952; Biggs *et al.*, 1952).

Hemophilia B is less frequent than hemophilia A, with an approximate a 1/8 or 1/10 ratio.

The hereditary transmission is sex linked. The FIX gene is located on the X chromosome not far from the gene controlling Factor VIII activation. Both sporadic and familial forms have been described.

The clinical picture is indistinguishable from that seen in hemophilia A. Hemarthroses, deep muscle hematomas, hematuria, and easy bruising are most frequent manifestations.

A. Classification and prevalence

Classification: the defect may be subdivided in severe (FIX activity <1% of normal), moderate (FIX activity, 1–5% of normal), and mild (FIX activity, 5–35% of normal). Mild forms may be asymptomatic; moderate forms usually bleed after surgery or trauma. Severe forms show spontaneous bleeding.

Contrary to what seen in hemophilia A, antigen levels may be absent (CRMneg), reduced (CRMred), or normal (CRMpos) (Denson *et al.*, 1968). Several variants have been described. The most important ones are FIX Leiden and FIX Bm. About 60% of patients show no CRM in their plasma. About 30% of the remaining 40% are of the Bm type.

The main feature of hemophilia B Leiden is a tendency to improve with age. The main feature of hemophilia Bm is to show an inhibitory effect on the FVII-tissue thromboplastin reaction when the latter is obtained from ox-brain or pig-brain. In other words, the Bm hemophilia behaves as *in vitro* “anticoagulant” or “inhibitor” in the prothrombin time system.

Recent molecular biology studies have supplied a lot of information, and now the number of mutations responsible for hemophilia B is countless.

In the past, another form of hemophilia B received some attention, namely, the form associated with mild Factor VII deficiency (Elodi, 1973; Elodi and Puskas, 1972; Girolami *et al.*, 1977a). However, the existence of such form has not been fully confirmed (Girolami *et al.*, 2007).

B. Hereditary transmission and genetics

The hereditary transmission is X-linked as hemophilia A. The carrier state is characterized by slightly decreased or borderline levels of FIX activity with normal or near normal FIX antigen.

The gene for FIX is located in the long arm of X chromosome, close to the Factor VIII gene.

It consists of eight exons and seven introns. Exons I and II encode for a pre-pro leader sequence and the Gla domain. Exon III, which is a very short one, is involved in the aromatic stock domain and the last γ -carboxyglutamic acid residue. Exons IV and V encode the EGF domain. Exons VI, VII, and VIII encode the activation peptide and the catalytic domain.

The number of chromosomal alterations reported in hemophilia B is very large, and therefore it is impossible to go into details. Tuddenham and

Cooper listed about 150 samples (Tuddenham and Cooper, 1994). Several hundred more have been added subsequently, bringing the total now to about 2,500 (database).

Briefly, mutations may be subdivided into: (1) deletions, (2) point mutations (both missense and nonsense), (3) insertions, and (4) inversions. The majority of missense mutations found in mild hemophilia B are not sporadic or *de novo*. On the contrary, the majority of mutations found in the moderate and severe hemophilia B are of the sporadic or the *de novo* type. A representative selection of these molecular alterations have been gathered in Tables 14.16 and 14.17.

All regions of FIX gene have been involved. Several mutations, localized in different areas, have given origin to severe hemophilia B. Among those involving the promoter region, FIX Leiden is worth of special mutation. The patient with FIX Leiden shows a tendency to improve with age. The defect consists in a single nucleotide substitution in an area of the promoter region (usually -21) and (Bezeaud *et al.*, 1984) sensitive to androgens (androgen responsive element or ARE) (Crossley *et al.*, 1992; Reijnen *et al.*, 1992, 1993; Reitsma *et al.*, 1988). Similar hemophilia B features have been described even with mutations in a wider area (-21 + 13 region) (Giannelli *et al.*, 1998, 1999; Triplett *et al.*, 1985). Astonishingly, mutations at -26 seem to disrupt completely the ARE, and therefore the hemophilia B patients carrying this mutation fail to improve with age. The promoter mutations responsible for hemophilia Bm variably alter the pattern of expression, whereby the FIX gene becomes transcriptionally

Table 14.16 Selected examples of mutations seen in some cases of hemophilia B without antigen (CRM negative)

References	Eponym n.r. or omitted (see text)	Mutation (Exon)	Phenotype
Ludwig <i>et al.</i> (1989)	—	Arg338 Stop (Exon VIII)	Severe
Attree <i>et al.</i> (1989)	—	Lys411 Stop (Exon VIII)	Severe
Green <i>et al.</i> (1989)	—	Asn120Tyr (Exon V)	Severe
Koeberl <i>et al.</i> (1990)	—	Gly60Ser (Exon IV)	Mild
Chen <i>et al.</i> (1991)	—	Trp215 Stop (Exon VII)	Severe
Winship and Dragon (1991)	—	Glu7Ala (Exon II)	Mild

This pattern may be seen with mutations in any area; n.r., not reported.

Table 14.17 Selected examples of mutations seen in some cases of hemophilia B with normal FIX antigen (CRM positive)

References	Eponym	Mutation (Exon)	Phenotype
Noyes <i>et al.</i> (1983)	Chapel Hill	Arg145Hys (Exon VI)	Mild
Davis <i>et al.</i> (1987)	Alabama	Asp47Gly (Exon IV)	Mild phenotype
Reitsma <i>et al.</i> (1988)	Leiden	Promoter	Androgen sensitive
Tsang <i>et al.</i> (1988)	London 2	Arg333Gln (Exon VIII)	Severe
Ware <i>et al.</i> (1989)	San Dimas	Arg -4 Gln (Exon II)	Severe phenotype
Bertina <i>et al.</i> (1990)	Novara	Arg180Gln (Exon V)	Bm type
Lozier <i>et al.</i> (1990)	New London	Glu50Pro (Exon IV)	Mild
Chen <i>et al.</i> (1991)	n.r.	Val107Ala (Exon V)	Mild

Both mild and severe phenotypes have been described. Patients with mutations in Exon VII apparently do not present normal or near normal antigen. On the contrary, mutations in Exons II or VI show frequently CRM. CRM, cross-reacting material.

active only after puberty (Reijnen *et al.*, 1992; Reitsma *et al.*, 1988). The underlying mechanism for this behavior seems because of a defective binding of this mutated FIX promoter to hepatocyte nuclear factor-4 (HNF-4) (Reijnen *et al.*, 1993).

It would seem that androgen hormones may somehow mitigate or even neutralize the defect. These patients note, after puberty, a decrease of the frequency and intensity of bleeding manifestations; FIX levels increase and even severe hemophiliacs may be changed in mild ones.

Another peculiar form of FIX deficiency is the hemophilia Bm. This defect, first described by Hougie and Twomey (1967), is characterized by the presence of a CRM that interferes, at least *in vitro*, with the TF + Factor VII reaction when certain slow thromboplastins, for example ox-brain or pig-brain thromboplastin, are used in the test. It is estimated that about 15% of patients with CRM show this phenomenon. This condition may sometimes create diagnostic difficulties as will be discussed later. It has been demonstrated that the inhibitory effect correlates with the level of antigen. The maximal prolongation of ox-brain thromboplastin is observed in those patients who have normal or even slightly increased level of FIX antigen. Patients with lower levels of antigen show less inhibition (Girolami *et al.*, 1982a).

The genetic alterations seen in this variant involve Exon VI or Exon VIII. They are all point mutations. The reason why mutations in two nondependent exons may yield the same phenotype is unknown and confirms what seen also for other clotting factors, namely, that the same phenotype may be seen with different mutations occurring even in different regions. Furthermore, it has to be noted that there is no absolute correlation between mutation and phenotype. For example, the same mutation (Ile397Thr) has been seen both in families with and without the Bm phenomenon (Tuddenham and Cooper, 1994).

Point mutations are by far the most frequent alterations: they represent in fact about 90% of all the changes. Deletions are present in about 6–7% of cases, while insertions and other changes are very rare. This is quite different from what occurs in hemophilia A, where Intron 22 inversion is frequently present in severe forms of the disease (Bowen, 2002).

Besides the promoter region, changes in all exons (from Exon II to Exon VIII) have been associated with hemophilia B phenotype. Mutations in Exon VII seem to cause only cases of hemophilia B without any CRM (true deficiency). Mutations in Exon VIII also seem to cause a prevalence of CRMneg forms. Residues 390–397 of Exon VIII seem critical in binding to Factor X and/or to antithrombin. Selected but representative lists of mutations causing CRMneg or CRMpos hemophilia B are reported in Tables 14.16 and 14.17. A detailed analysis of different molecular alterations seen in FIX deficiency is beyond the scope of this clinical review that must deal mainly with the phenotype.

Hemophilia B has occasionally been described in females (Costa *et al.*, 2000; Di Paola *et al.*, 2004; Sellner and Price, 2005; Shetty *et al.*, 2001a; Spinelli *et al.*, 1976; Yang and Ragni, 2004). The molecular alterations seen in these patients are: (1) homozygosity for hemophilia gene (very rare), (2) nonrandom or skewed inactivity of X chromosome, (3) double *de novo* mutation, (4) mosaicism, (5) deletion of tip of long arm of X chromosome, (6) translocations, (7) segmental isodisomy, and (8) other structural abnormalities of X chromosome. Mosaicism may sometimes be associated with Turner's syndrome (45, X0).

Bleeding manifestations are usually less severe than those seen in classical hemophilia B patients.

FIX levels are variable, but very low levels as those seen in severe hemophilia B (around 1% of normal) are rare. Diagnosis may be difficult, since one does not expect to be facing a woman with hemophilia B.

C. Biochemistry and function

Factor IX is a single chain vitamin K-dependent glycoprotein with a molecular weight of about 57,000 Da.

It is constituted of 415 amino acids. Its structure is similar to that of other vitamin K-dependent factors. It contains several domains, namely, a 12 γ -carboxyglutamic acid residue domain located in the amino-terminal end; two EGF-like domains are also located in this part of the protein. Finally, there is an activation peptide together with the catalytic domain. It circulates at an approximate concentration of 0.4 mg%.

FIX is activated into FIXa by cleaving two arginine peptide bonds (Arg145 and Arg180). The result of such activation is the formation of a light chain (catalytic area) and a heavy chain held together by disulfide bonds together with an activation peptide. Activated FIX in the presence of phospholipids, Ca^{2+} , and Factor VIII forms a prothrombinase that activates Factor X to Factor Xa.

The main types of functional and/or biochemical defects at the base of hemophilia B could be summarized along the following lines: (1) defects with abnormal carboxylation, (2) cases with reduced activation from Factor XIa, (3) Bm type, (4) alteration in the catalytic area, and (5) changes in the androgen sensitive area ([Attree et al., 1989](#); [Bachmann et al., 1958](#); [Bicocchi et al., 2006](#); [Chen et al., 1991](#); [Davis et al., 1987](#); [Green et al., 1989](#); [Koeberl et al., 1990](#); [Lozier et al., 1990](#); [Ludwig et al., 1989, 1992](#); [Mukherjee et al., 2003](#); [Noyes et al., 1983](#); [Odom et al., 1994](#); [Spitzer et al., 1990](#); [Taylor et al., 1992](#); [Tsang et al., 1988](#); [Usharani et al., 1985](#); [Ware et al., 1989](#); [Winship and Dragon, 1991](#)).

D. Clinical picture

The clinical picture is similar to that of hemophilia A. No differentiation of the two forms is possible on clinical presentation.

The first clinical manifestation usually occurs in infancy because of ecchymosis and hematomas secondary to accidental falls. Hemarthroses may occur after a few years. These are posttraumatic at the beginning, later on they occur spontaneously. Patients with hemophilia B Leiden show an attenuation of the bleeding diathesis after puberty ([Crossley et al., 1992](#)).

Easy bruising and superficial or deep muscle hematomas are frequent. As in hemophilia A, hematomas of the ileopsoas may occur. Hematuria is also frequent. Brain hemorrhage, as in other prothrombin factors deficiencies, may be the cause of death. Moderate forms with FIX levels between 2% and 5% are paucisymptomatic. Hemarthroses usually occur only after traumas. Mild forms (FIX level >5%) are usually asymptomatic. These patients usually bleed excessively only after surgery or trauma.

Carriers are asymptomatic. The same is true for mild forms that, unless challenged by trauma or surgery, may remain asymptomatic. The appearance of an inhibitor, which is approximately as frequent as in hemophilia A, aggravates the bleeding manifestations.

Women with hemophilia B are variably symptomatic according to the level of FIX. The cases due to skewed or nonrandom inactivation are usually less severe, equivalent to males with mild hemophilia and therefore show a limited bleeding tendency (Lusher and McMillan, 1978).

E. Diagnosis

There is no possibility to diagnose hemophilia B on clinical grounds. The laboratory criteria for the diagnosis of hemophilia B are: prolonged PTT, usually normal PT, and normal TT. FIX assay varies between 1% and 25% of normal, allowing the distinction between severe, moderate, and mild forms. There is a good correlation between FIX levels and clinical phenotype. The prolonged PTT is corrected by the addition of normal plasma, normal serum but not by the addition of adsorbed normal plasma. In the case of hemophilia Bm, prothrombin time is prolonged, particularly if one uses ox-brain thromboplastins such is the case with Thrombotest (Owren, 1959) (Table 14.18). The prolongation is variable but may be marked (ex 100 s vs. a normal of 40 s). Interestingly, such prolonged PT is not corrected by the addition of normal plasma, contrary to what happens for the PTT. As a matter of fact, in serial dilutions and cross-studies, it has been demonstrated that even 10–20 of the patient's plasma in a mixture with normal plasma is able to prolong slightly the ox-brain thromboplastin clotting time (Fig. 14.5). Furthermore, there is a correlation between the level of antigen or of the difference of antigen versus activity and the degree of the ox-brain thromboplastin prothrombin time (Fig. 14.6). Factor IX antigen is absent in about

Table 14.18 Behavior of clotting global tests in the differential diagnosis of Factor VII (FVII), Factor IX (FIX), and Factor X (FX) deficiencies and some variants

Defect	PTT	PT rabbit- brain	PT ox- brain	RVV	Comments
Classical FIX Def. (hemophilia B)	P	N	N	N	
Hemophilia Bm	P	N (1)	P	N	(1) It may be 1–2 s prolonged
Classical FX Def.	P	P	P	P	
FX Friuli	P	P	P	N	
FX Padua (Riadh)	N	P	P	N	
FX Melbourne	P	N	N	N	
Classical FVII Def.	N	P	P	N	
FVII Padua	N	P	N	N	

P, prolonged; N, normal; RVV, Roussell viper venom.

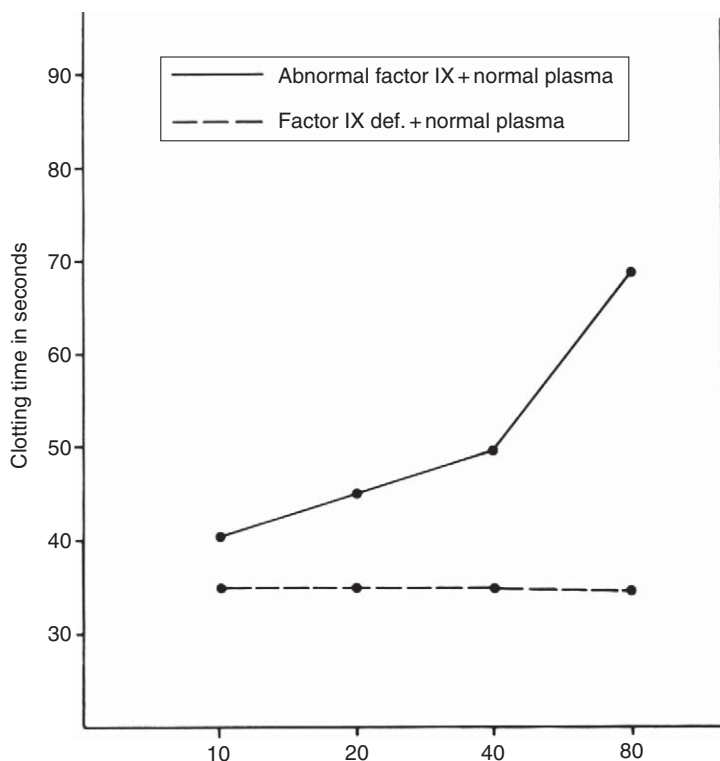


Figure 14.5 Inhibitory effect of hemophilia Bm plasma on ox-brain thromboplastin prothrombin time (Thrombotest). Even the addition to normal plasma of 10–20% of hemophilia Bm plasma causes a prolongation of the Thrombotest. On the contrary, the addition to normal plasma of increasing aliquots of classical hemophilia B [cross-reacting material (CRM) negative] remains without effect. Percentile parts of patient plasma in abscissa.

50% of patients. In the remaining 50%, it is variably present in a reduced or normal form. Cases with normal or near normal FIX antigen are about 30% of the total.

In case of hemophilia Bm (about 20% of cases with CRM), FIX antigen is always present even though to a variable extent (50–100% of normal). Carriers show borderline or slightly decrease FIX activity and, usually, normal FIX antigen.

Molecular biology analyses allow a more precise diagnosis but are not needed in routine clinical practice. The two most important variants, hemophilia B Leiden and hemophilia Bm, can be diagnosed by clinical and specific clotting test. Detection of carriers used to be carried out by clotting versus antigen evaluation. Recently, molecular biology techniques are widely used: DNA polymorphisms analysis, screening procedures such

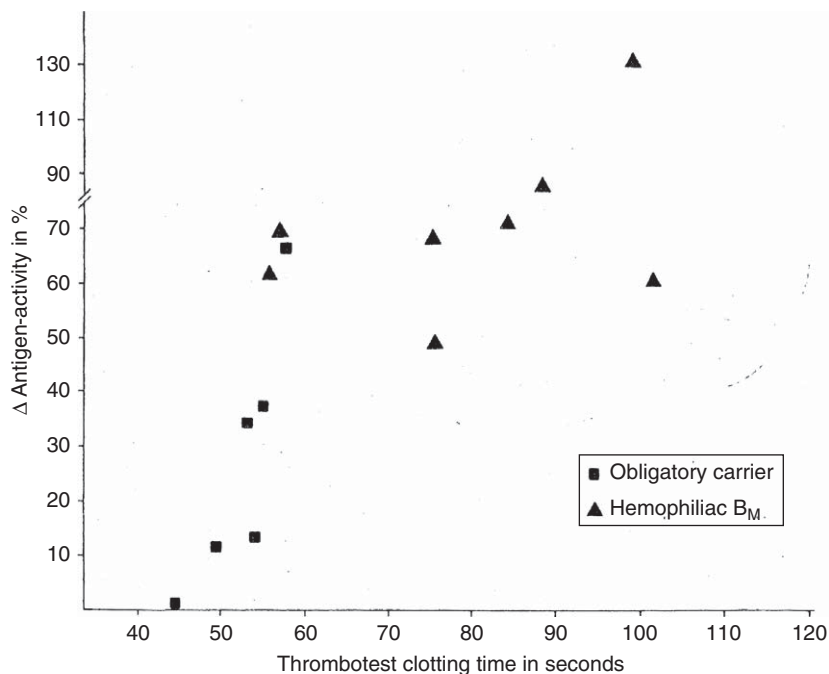


Figure 14.6 Correlation between Thrombotest clotting times (*abscissa*) and antigen-activity difference (*ordinate*). Besides the nine hemophilia B_M patients, available mothers or daughters of hemophiliacs (obligatory carriers) were also included. The mother of two known hemophiliacs, who were dead at the time of the study, is also included as an obligatory carrier. Note that the only patient with a normal Thrombotest had a Δ antigen-activity equal to zero. The correlation is significant ($r = + 0.78$; $t = 4.30$; $p < 0.001$).

as conformation sensitive gel electrophoresis (CSGE), and direct mutation detection (Ljung, 1969).

Prenatal diagnosis has been carried out by the same or similar techniques on material obtained by amniocentesis or chorionic villi biopsy. Factor IX assay on blood obtained by cordocentesis has also been useful in some cases (Shetty and Ghosh, 2007). Finally, direct analysis of fetal DNA circulating in maternal blood has been proposed (Costa *et al.*, 2002).

F. Prognosis

Prognosis is guarded in cases of severe forms. Brain hemorrhage is a dreadful complication as in the deficiencies of the other PC factors. Data on survival are scanty. It has surely improved with the widespread use of concentrates,

but this beneficial effect has often been wiped out by the epidemic of blood transfusion-transmitted diseases. In recent years, this negative effect has faded and survival rates are increasing again. It has been estimated, in fact, that life expectancy for severe hemophilia patients without HIV infection is around 60 years, with no significant differences between hemophilia A and B (Darby *et al.*, 2007).

G. Therapy

Several variably purified concentrates, including recombinant FIX ones, are available for therapy (Kessler, 2005). Half-life is about 15 h, and safe hemostatic levels are around 30–40% of normal. This is based on the observation that carriers with a FIX level of about 40–50% of normal are asymptomatic.

Prothrombin complex concentrates were widely used in the past but have now been almost abandoned because of the high risk of viral contamination and of thrombotic complication due to the frequent presence in such concentrates of activated clotting factors. Recombinant FIX represents now the treatment of choice (Kessler, 2005; Roth *et al.*, 2001). Recombinant-activated FVII has also been used in critical conditions, but this is also associated with the high risk of thrombosis and should be discouraged (O'Connell *et al.*, 2006) unless inhibitors are present.

Antifibrinolytic drugs have been used as an adjunct to substitution therapy. Prophylaxis regimens with recombinant FIX are now used with 500 units twice a week for a 70–80 kg male. Development of inhibitors creates a severe therapeutic problem (Dimichele, 2007). Inhibitors develop in about 2% of hemophilia B patients. Since hemophilia B is at least six times less frequent than hemophilia A, the problem has, apparently, a smaller impact. However, this may be a fallacious impression due to the fact that the treatment of hemophilia B patients with inhibitors has so far less options than for patients with hemophilia A. Immune-tolerance results, for example, appear to be less favorable (Dimichele, 2007; Soto *et al.*, 2004). Activated PC concentrates or recombinant-activated FVII concentrate are the most widely used therapeutic tools (Dimichele, 2007). Immunoadsorption, cyclophosphamide, and cortisone have also been occasionally used with variable success.

Liver transplant has been carried out in a few instances, with apparent fair or good results, obtaining protracted normal or hemostatically satisfactory FIX levels (Johnston *et al.*, 1998; Kadry *et al.*, 1995; Kayler *et al.*, 2002; McCarthy *et al.*, 1996; Merion *et al.*, 1988; Sugawara *et al.*, 1997; Wilde *et al.*, 2002). The follow-up has been satisfactory in a few instances for 2–3 years. The procedure also appears to slow HIV progression. This approach is usually reserved for patients with hemophilia and liver failure due to hepatitis (Table 14.19) (Gordon *et al.*, 1998).

Table 14.19 Cases of liver transplantation in hemophilia B patients reported in the literature

References	Age of transplant	Type of hemophilia	Type of transplant	Type of hepatitis	HIV infection	Outcome	Comments
<i>Merion et al.</i> (1988)	60	Mild	Orthotopic	B	No	Good	
<i>Kadry et al.</i> (1995)	44	Severe	Orthotopic	B/C	No	Fair	Survival for 2 years
<i>McCarthy et al.</i> (1996)	40	Severe	Orthotopic	C	No	Poor	Death 20 days after transplant
<i>Johnston et al.</i> (1998)	60	Mild	Orthotopic	C	No	Good	
<i>Kayler et al.</i> (2002)	14	Severe	Orthotopic	n.r.	n.r.	Good	30 months of follow-up
<i>Sugawara et al.</i> (2002)	41	Severe	Living donor	C	Yes	Good	Delayed HIV disease progression
<i>Wilde et al.</i> (2002)							Acute rejection.
Case 1	52	Severe	Orthotopic	C	Yes	Fair	Immunosuppressive therapy
Case 2	59	Moderate	Orthotopic	B	No	Poor	Acute renal failure

Gene therapy is also being investigated. Several vectors have been attempted, but the results obtained have been meager so far.

In a few studies it was employed, AAV mediated FIX gene transfer to skeletal muscle. Minimal and short-lived elevations of FIX were obtained in some of the patients investigated (Jiang *et al.*, 2006; Manno *et al.*, 2006). However, in one study enrolment was stopped because of increase in transaminases levels. Altogether, gene therapy for hemophilia B remains so far only a theoretical proposal (McCarthy *et al.*, 1996; Ponder, 2006).

H. Combined defects of factor IX

FIX defect, hemophilia B, has been rarely described in association with Factor VIII deficiency (hemophilia A). The condition is due to the presence, in the same woman, of a double carrier state for hemophilia A and B. The number of cases reported is limited, around 20 cases (Robertson and Trueman, 1964; Shetty *et al.*, 2001b).

The clinical picture is similar to that of severe hemophilia A or B.

The main features are a prolonged PTT partially but not completely corrected either by the addition of normal serum or by adsorbed normal plasma.

Another association that has obtained great attention is that with Factor VII (Battin *et al.*, 1988; Bell and Alton, 1955; Constandoulakis, 1958). At least three conditions characterized by combined FIX and Factor VIII deficiency have been reported, namely, (1) the result of a casual association between hemophilia B with FVII deficiency. A few families have been described where the two defects segregate independently; (2) the result of laboratory artifact as seen especially in hemophilia Bm due to interference of the abnormal Factor IX in the Factor VII assay. This effect may be mild and go unnoticed when rapid tissue thromboplastins (rabbit or human brain) are used in the assay system, but may be evident when slow thromboplastins (ox-brain) are used; (3) the existence of a combined FIX and Factor VII defect due to a common cause. This last possibility remains to be proven. Needless to say that the combined defect of all PC factors is a separate clinical entity that will be treated separately.

V. CONGENITAL FACTOR X DEFICIENCY

Since Factor X (FX) plays an important role in blood coagulation, it is not surprising that the discovering of a congenital deficiency of this factor represented a major event in blood coagulation.

The main problem concerned the differences from Factor VII deficiency, which, being less rare, was described before FX, namely, in 1951.

Such separation between FVII and FX deficiency with the description of the first cases of isolated congenital FX deficiency was accomplished in 1956 and 1957 with the description of the three kindreds (Power in England, Stuart in USA, and another in Switzerland). Several cases have been added afterward.

Note that the classical FX deficiency in the Stuart family had been described before as a case of hypoconvertinemia (Graham, 2003). The same was true for two Factor X Friuli patients (Girolami *et al.*, 1970), who were first described as cases of FVII deficiency and subsequently, after the description of FX deficiency, the patients were restudied and described as cases of FX deficiency (Chevallier *et al.*, 1955, 1959). This represented an improvement but the final diagnosis of an abnormal FX arrived later when anti-FX antiserum became available (Girolami *et al.*, 1970). Several reviews have appeared during the past decade (Cooper *et al.*, 1997b; James, 1994; Perry, 1997; Uprichard and Perry, 2002). A great heterogeneity of the defect has been demonstrated (Denson *et al.*, 1970; Fair and Edgington, 1985).

A. Classification and prevalence

Factor X defects should be classified as: (1) “true” deficiency, (2) FX abnormalities, and (3) combined defects. This classification is based on both activity and antigen assays. Families investigated only on activity assays cannot be adequately evaluated even if molecular biology data are available (Herrmann *et al.*, 2006).

Similarly to what already seen with other factors of the PC, the patients with “true” deficiency show a parallel decrease in FX activity and antigen. This form is also called Type I.

Patients with abnormal FX show low activity and slightly decreased or even-normal antigen, the so-called Type II. The cases with normal FX antigen are usually referred to as CRMpos, those with intermediate levels as CRMred, whereas the cases with “true” deficiency are defined as CRMneg. Approximately 1/3 of patients are cases of true deficiency; in remaining 2/3, FX antigen is present, either in normal or in reduced quantity. On the basis of cases so far described, a simple classification has been proposed (Girolami, 1986): true deficiency (Stuart defect) (Graham *et al.*, 1957; Hougie *et al.*, 1957), cases with low activity and normal antigen (Prower defect) (Blanchette *et al.*, 1991; Cooper *et al.*, 1997b; Denson *et al.*, 1970), forms with normal RVV (Friuli defect) (Girolami, 1971; Girolami *et al.*, 1971; James *et al.*, 1991; Kim *et al.*, 1996) and forms with defect only in the extrinsic system (Padua and similar defects) (Al-Hilali *et al.*, 2007; Bertina *et al.*, 1981; Nora *et al.*, 1985; Parkin *et al.*, 1974), and forms with a defect only in the intrinsic system (FX Melbourne) (Parkin *et al.*, 1974) (Table 14.20).

These five types represent the index patients who have a distinct laboratory pattern and variable clinical picture. The majority of cases described in

Table 14.20 Tentative classification of Factor X deficiency and paradigmatic defects

Condition	Factor X assay					
	Tissue thromboplastins (extrinsic)	RVV	Cephalin (intrinsic)	Chromogenic	Immunological assay	Bleeding manifestations
Classic FX Def. (Mr. Stuart) ^a	Low	Low	Low	Low	Low	Severe
Miss Prower Def. ^b	Low	Low	Low	?	Normal	Severe
FX Friuli Def. ^c	Low	Normal	Low	Low	Normal	Moderate
FX Melbourne	Normal	Normal	Low	?	Normal	Moderate
FX Padua ^d	Low	Normal	Normal	Low	Normal	None

^{a,b} Several similar cases described.

^c No similar cases reported.

^d A few additional similar cases reported (FX San Antonio, FX Riyadh).

recent years by molecular biology techniques fit either the Stuart or the Prower genotype. In other words, they are either cases of “true” deficiency with no CRM or cases with variable levels of CRM on their plasma (Bernardi *et al.*, 1989; Bezeaud *et al.*, 1995; Fair *et al.*, 1989; Forberg *et al.*, 2000; Girolami *et al.*, 1976; Hayashi *et al.*, 1998; Herrmann *et al.*, 2006; Huisse *et al.*, 1985; Kim *et al.*, 1996; Marchetti *et al.*, 1995; Miyata *et al.*, 1998a; Peyvandi *et al.*, 2002; Pina-Cabral and Justica, 1973; Simioni *et al.*, 2001; Watzke *et al.*, 1990; Zama *et al.*, 1999).

Combined defects refer to cases of Factor X defect in association with other clotting defects.

The estimated prevalence of FX deficiency is about 1/1,500,000 or 2,000,000, therefore the condition to be considered as one of the rarest coagulation disorders.

B. Biochemistry and function

FX is a vitamin K-dependent glycoprotein, synthesized in the liver as a single chain. It circulates as a two chain at a concentration of about 1 mg%.

Its structure is similar to that of other PC clotting factors. The circulating double-chain form consists of a light chain of 139 amino acids and a heavy chain of 346 residues bound by disulfide and other bonds (Hertzberg, 1994; James, 1994).

The light chain contains three structural domains, whereas the heavy chain contains the serine protease domain that forms the catalytic triad of the active site.

The activation of FX occurs by the cleavage of the Arg194–Ile195 bond of the heavy chain. Such activation occurs physiologically either by the intrinsic system (FIXa and Factor VIIIa, phospholipids, Ca^{2+} ions) or by the extrinsic system (TF, FVIIa, Ca^{2+} , and phospholipid membrane). Nonphysiological activation is represented by RVV because of the presence in this venom of a metalloproteinase. The main substrate of FXa is prothrombin; other minor substrates are Factors V and FVII.

The FXa activity is regulated by natural occurring inhibitors, namely, antithrombin, which is a direct inhibitor. An indirect inhibitor may be considered the TFPI. This inhibitor forms a quaternary complex with TF, FVIIa, and FXa devoid of any activity. Recently, an inhibitory effect on FXa has been proposed also for the protein Z-dependent protease inhibitor (Broze, 2006). However, its significance remains to be clarified.

C. Hereditary transmission and molecular genetics

The hereditary transmission of FX deficiency is autosomal recessive. People who are homozygous for true deficiency have very low FX levels and equivalently depressed FX antigen.

Those who are heterozygous have FX levels of about 50% of normal levels both as activity and as antigen.

In homozygous FX abnormalities, FX activity level is variably depressed, usually around 5–15% of normal, while normal or near normal antigen is present. Heterozygotes, in this case, have activity of about 50–60% of normal and normal FX antigen levels. In compound heterozygotes (hypo-dys or dys-dys), FX levels are variable. For example in the association of a true deficiency with an abnormality (hypo-dys form), FX level is usually around 50–60% of normal both as activity and as antigen (Fig. 14.7).

In compound heterozygous, the defects segregate independently in the family so that some family members may have only one of the two defects.

The human FX gene is 22-kb long and is located at 13q34-ter, <3 kb from FVII. The sequence of human FX gene is highly homologous to other vitamin K-dependent coagulation factors such as FVII, FIX, and Protein C, suggesting its origin from a common ancestral gene. The coding region of FX is divided into eight exons, each encoding for a particular domain within the protein. Exon I encodes the signal peptide, Exon II the propeptide and the Gla domain, and Exon III a short-linking segment of aromatic amino acids. Exons IV and V encode the two EGF domains; Exon VI encodes the activation peptide at the N-terminal of the heavy chain; and Exons VII and VIII encode the serine protease domain with the catalytic triad His236, Asp228, and Ser379 (Table 14.21).

Although the clinical presentation of FX deficiency correlates fairly well with laboratory phenotype, genetic analysis may sometimes provide a further explanation for the FX activity and antigen levels measured in the laboratory. As in the other congenital coagulation disorders, FX deficiency usually arises from missense mutations. Amino acid substitutions can significantly change any number of steps in protein formation. Mutations severely impairing the protein structure are frequently associated with a reduction in antigen and consequently in the activity level. In these cases, abnormal protein folding may be responsible for an impairment of protein secretion and/or a reduction in protein half-life. Missense mutations responsible for minor structural changes are often associated with an impairment in FX activity in the setting of a normal antigen level. Large gene deletions have been reported and are invariably associated with a symptomatic pattern when found in compound heterozygosis with a missense mutation impairing the FX protein structure. Very few symptomatic individuals with total or severe deficiency in the components of prothrombinase have been identified to date (Cooper *et al.*, 1997b; Millar *et al.*, 2000; Oldenburg *et al.*, 2007). It is likely that genetic mutations that cause total FX deficiency in humans may often be incompatible with life. Missense mutations have been described affecting the different FX domains. Mutations in the preprosequence and prosequence, as FX Nice and FX Santo Domingo, have been shown by molecular modeling and expression studies to be responsible

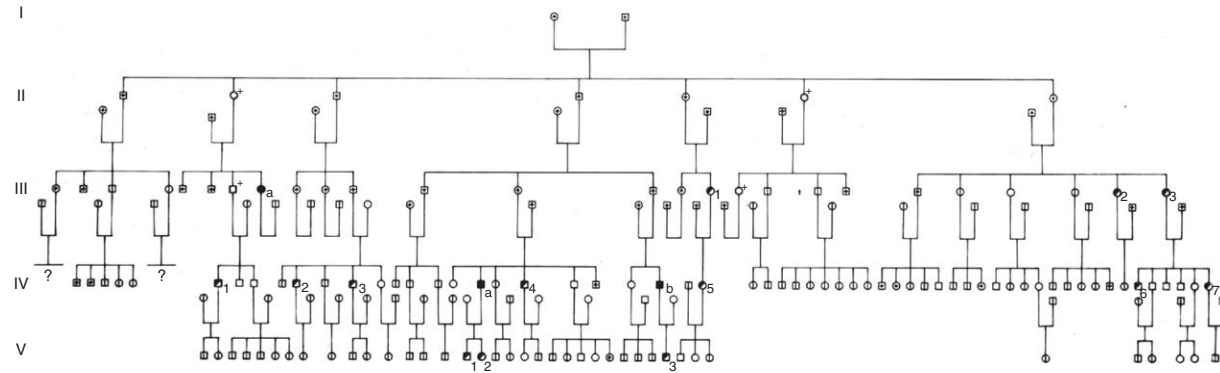


Figure 14.7 The FX Friuli index kindred. The hereditary transmission is autosomal recessive. Consanguinity is evident. Homozygotes are IIIa (index patient) and her two relatives (IVa and Vb). Numerous heterozygotes are present in the family tree. Classical FX deficiency has the same hereditary pattern. Symbols have the same meaning as in [Fig. 14.1](#).

Table 14.21 Main features of some representative patients with variably severe “true” FX deficiency. Eponyms have been disregarded

References	FX activity				FX Antigen	Bleeding tendency	Genotype	Mutation
	Intrinsic	Extrinsic	RVV	Chromogen (S2222)				
Graham <i>et al.</i> (1957) and Cooper <i>et al.</i> (1997b)	<1	<1	Low	n.r.	Traces	Severe	Hom.	Stuart pedigree
Watzke <i>et al.</i> (1991)	1	<1	n.r.	n.r.	<5	Severe	Hom.	Val 289 Met
Miyata <i>et al.</i> (1998a)	n.r.	3	n.r.	n.r.	<10	Moderate	Hom.	Gly -20 Arg
Miyata <i>et al.</i> (1998b)	n.r.	4	n.r.	n.r.	7	Moderate	Comp. Het.	Arg 306 Cys
Hayashi <i>et al.</i> (1998)	n.r.	25	4.5	n.r.	<5%	Moderate	Hom.	Met 40 Val
Forberg <i>et al.</i> (2000)	30	1	18	n.r.	20	Moderate	Double Hom.	+ Pro 204 Ser
Peyvandi <i>et al.</i> (2002)								Triplet deletion intron D
H	<1	2	1.5	9	4	Severe	Hom.	Gla 129 Lys
J	<1	<1	<1	3	10	Severe	Hom.	+ Glu 102 Lys
Vianello <i>et al.</i> (2003)	4	4.7	3.9	2.5	5	Moderate	Hom.	Gly 94 Arg
								Gly 222 Arg
								Cys 350 Arg

List is obviously incomplete.

Letters are those proposed by authors to define their families. RVV, Roussel viper venom; n.r., not reported; Hom., Homozygote; Comp. Het., Compound heterozygote; Het., Heterozygote.

for secretion and cleavage problems explaining the Type I phenotype (Miyata *et al.*, 1998b; Watzke *et al.*, 1991).

A missense mutation affecting the calcium-binding region of the Gla domain characterizes the low activity and normal antigen level in the FX St. Louis (Rudolph *et al.*, 1996). Mutations affecting the correct formation of disulfide bridges in EGF-1 and EGF-2 domain have been reported, affecting both the function and the antigen level of FX-associated mutations. Missense mutations affecting the release of the activation peptide, such as FX Wenatchee I and II, have been shown to be associated with low-catalytic domain. This variant consists of a Pro343Ser substitution within the heavy chain of FX (Kim *et al.*, 1995). Homozygous FX Friuli patients present with a normal antigen level and a normal or near normal activity by means of RVV assay, but a severely reduced (4–9%) function in the intrinsic and extrinsic pathways. Because of the formation of a new hydrogen bond, the tertiary structure of the catalytic domain has been shown to be affected. A good genotype–phenotype correlation has been demonstrated in an Italian family with FX deficiency, named FX S. Giovanni Rotondo (Simioni *et al.*, 2001). In this variant, one deletion in the region encoding for the activation peptide was described in compound heterozygosis with a missense mutation in the catalytic domain, respectively, responsible for the lack of synthesis and/or secretion of FX and for a dysfunctional FX (Table 14.22). The loss of a disulfide bond in the catalytic domain has been shown to be associated with a Type I phenotype in FX Padua 3. These few examples of a consistent genotype–phenotype relationship further suggest that the laboratory phenotype is largely a function of the FX gene lesion segregating in the family. Some variability, even in the presence of identical pathological FX genotypes, may be present, due to variation between laboratory assays, the effect of polymorphisms, and possibly also the influence of variation in nonallelic loci. In other cases, the explanation for these discrepancies remains unknown. In this regard, it is worth remembering that the FX variants with a defect only or predominantly in the extrinsic system (FX Padua, FX Riadh, and FX San Antonio) show different mutations, namely, Arg251Trp, Glu51Lys, or Arg326Lys + 272 Stop, respectively (Al-Hilali *et al.*, 2007; Girolami *et al.*, 1985b, 2004; Reddy *et al.*, 1989). Another example of the lack of a constant relation between genotype and phenotype. FX Roma (De Stefano *et al.*, 1988; Odom *et al.*, 1994) shows a pattern similar to that of FX Friuli (Girolami *et al.*, 1970; James *et al.*, 1991), namely, prolonged PTT and PT and normal RVV and antigen. This could suggest that the area of Exon 8 between codon 318 (Roma) and 343 (Friuli) is involved in the activation via the RVV. However, this is not absolute, since the Ser334Pro mutation (FX Marseille) shows a prolonged RVV clotting time (Bezeaud *et al.*, 1995). Another discrepancy that makes it so far very difficult to draw sure genotype–phenotype conclusions.

Table 14.22 Main features of the 5 FX abnormalities seen in Padua during the past 37 years. Cases with true deficiencies have been excluded

Abnormality	FX activity				Antigen	Bleeding	Genotype	Mutation and comments
	Extrinsic	Intrinsic	RVV	Chromogen (S2222)				
FX Friuli	8	6	90	2	100	Moderate	Hom. and Het.	Pro343Ser
FX Padua 1	76	90	88	20	100	Mild	Hom. and Het.	Arg251Trp
FX Padua 2	52	60	56	40	96	Moderate	Het.	Molecular biology studies not available.
FX San Giovanni Rotondo	9	24	43	22	72	Moderate	Comp. Het.	Lys408Pro microdeletion C556
FX Padua 3	8.2	19	12	10	40	No	Comp. Het.	Gly 380 Arg Ser 334 Pro ^a

^a First described in FX Marseille. RVV, Roussell viper venom; Hom., homozygote; Comp. Het., compound heterozygote; Het., heterozygote.

Expression studies, which allow to obtain recombinant forms of the FX been investigated, are essential to confirm that the mutation observed is indeed responsible for the defect. These studies are particularly indicated when dealing with abnormalities but, unfortunately, have been carried out only in a few cases (Forberg *et al.*, 2000; Kim *et al.*, 1996; Racchi *et al.*, 1993).

Molecular studies have recently allowed the construction of putative models that may help in understanding the structure–function relation.

D. Clinical picture

Among patients with rare coagulation defects, FX deficiency may be one of the most severe. The onset of hemorrhagic manifestations in marked deficiency is typically seen in infancy. In less severe deficiency, patients may start experiencing bleeding at any age. The most frequent sites of bleeding are the mucosal, soft tissue, and central nervous system. Easy bruising is also frequent. Among mucosal bleeding, epistaxis is the most frequent, followed by menorrhagia and gastrointestinal episodes. Soft tissues and joint bleeding such as muscle hematomas and hemarthroses may mimic classic hemophilia but are rare. Central nervous system hemorrhages represent life-threatening manifestations of severe FX deficiency. This is a relatively frequent cause of death as in other defects of the PC. It seems almost a specific symptom of the condition. Another severe bleeding complication is represented by hemoperitoneum associated with ovulation (Dafopoulos *et al.*, 2003).

There is no specific hemorrhagic pattern, and no sure diagnosis can surely be established on clinical grounds. Altogether, the picture is fairly similar to that of FII or FVII deficiency. Individuals with mild FX deficiencies may experience bleeding only after trauma or surgery.

Heterozygotes with FX levels around 50% of normal are usually asymptomatic but may present sometimes, contrary to what seen in heterozygotes for FVII deficiency, with easy bruising and bleed during surgery, traumas, and deliveries.

E. Diagnosis

Clinical signs and symptoms are not specific. They may vary in intensity according to the plasma levels. Diagnosis of FX deficiency can be reached only by specific laboratory investigation. The main features are: prolonged PTT PT with normal TT. RVV clotting time is also prolonged in the majority of cases, but it may be normal in some variants (FX Friuli). FX assays have to be carried out using at least three activation systems, namely, intrinsic, extrinsic, and RVV (Bachmann *et al.*, 1958; Girolami *et al.*, 1970). This is necessary because variants in which only one or two of these assays were abnormal have been described. FX Padua, for example, yielded a defective pattern only in the extrinsic system (Girolami *et al.*, 1985b, 2004). A few other cases with similar clotting features have been described (FX San

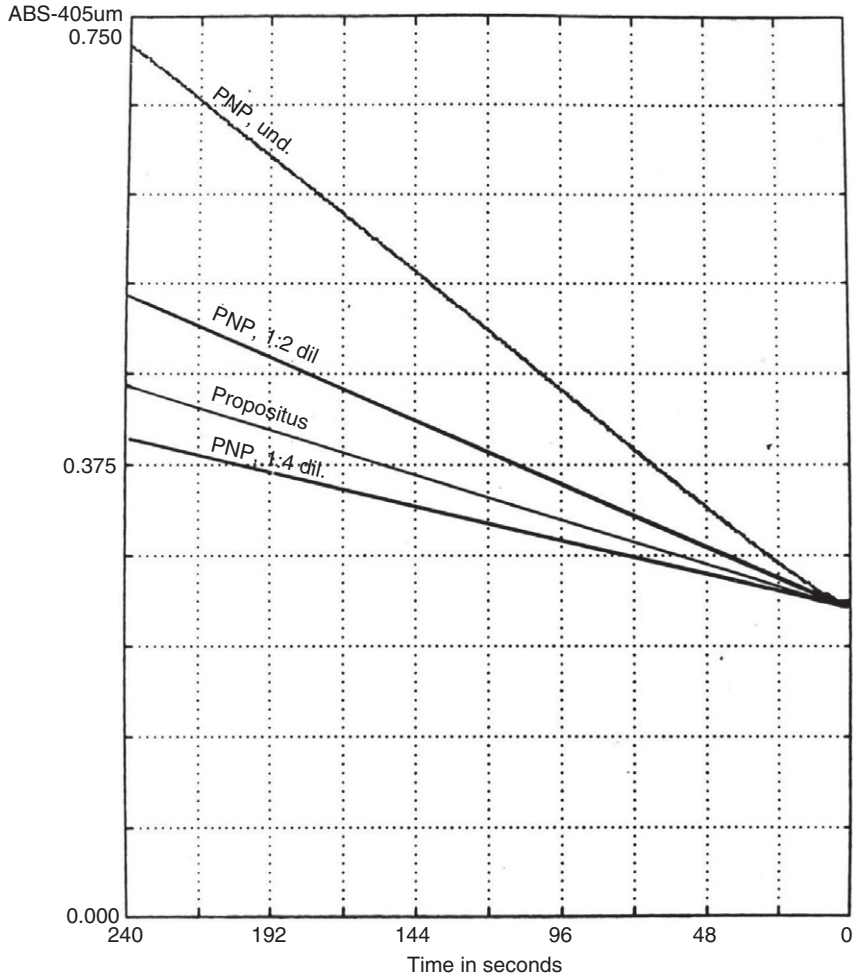


Figure 14.8 Chromogenic assay (S222). The propositus (a case of FX Padua 2) showed a level of about 38% of normal. Antigen level in this patient was 100% of normal.

Antonio and FX Riyadh) (Al-Hilali *et al.*, 2007; Reddy *et al.*, 1989). Chromogenic assays may also be useful since, sometimes, they may yield different levels of activity as compared with the clotting counterparts (Girolami *et al.*, 1980a) (Fig. 14.8).

These peculiar behaviors complicate the differential diagnosis in comparison with FVII deficiency. This is more so when one deals with cases of multiple factors deficiency, for example, combined Factor VII and Factor X defect (Table 14.20).

An immunological assay (usually, electroimmunoassay, radial immunodiffusion, ELISA, or laser nephelometry) is also indicated to find out if there is a discrepancy between activity and antigen level. In true deficiency, there is no discrepancy (CRMneg). In other forms, there may be an excess without reaching normal levels of antigen and activity (CRMred) (Girolami *et al.*, 1971; Mancini *et al.*, 1965; Vianello *et al.*, 2001, 2003).

When FX activity is low and FX antigen is normal or near normal, we have the so-called CRMpos. Genetic analysis does not add substantial information about diagnosis and clinical picture but is important for structure–function studies.

Molecular diagnosis of congenital FX deficiency relies on the detection of gene mutation and on the possible genotype–phenotype correlation. This can be achieved by amplifying the entire coding of the gene by PCR and then proceeding to sequence the PCR products using the appropriate primers (Fig. 14.9). Even with the introduction of automated sequencing system, some intermediate steps can be useful for scanning PCR products with the aim to identify the gene fragment potentially mutated and then confirm the mutation by sequencing (Fig. 14.10). The scanning techniques most commonly used for PCR products are single strand conformation polymorphism (SSCP), enzymatic or chemical cleavage of mismatched base pairs, and differential unfolding of homoduplex and heteroduplex by denaturing gradient gel electrophoresis (DGGE). Recently, CSGE has been successfully used as a simple, inexpensive, and highly reliable method for localizing FX mutations (Fig. 14.11). The combination of PCR CSGE with sequencing could be useful in family studies where, after complete characterization of the mutation in a family member by sequencing the entire FX gene, the other relatives can be screened by means of CSGE only (Vianello *et al.*, 2002). Although expression studies are invaluable to detect subtle functional differences in the cases where the mutations are antigen positive (i.e., normal antigen levels), they are less useful where the mutations are antigen negative (as is reflected in the low *in vivo* plasma levels), as the mutant proteins are likely to be unstable or may not be expressed. With the availability of FX crystal structures, sequence alignments and molecular modeling are effective methods in identifying structural perturbations in active sites (Vianello *et al.*, 2003). Molecular modeling in combination with energy refinements can be invaluable tools in the assessment of cases in which protein misfolding is the most likely outcome of a given mutation (Fig. 14.12). Molecular biology techniques have allowed a prenatal diagnosis in a few cases (Ingerslev *et al.*, 2007). In the context of a family with homozygotes, heterozygotes can easily be detected by repeated FX assays. There is usually little overlap, if any, between low normals and heterozygotes (Fig. 14.13) (Girolami *et al.*, 1974c). When dealing with sporadic cases, diagnosis may be more difficult.

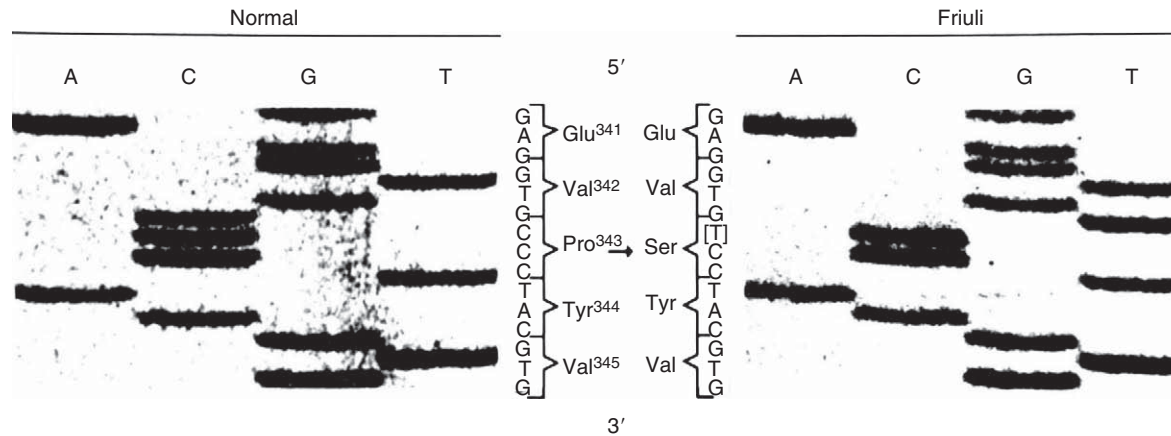


Figure 14.9 Autoradiographs of Exon VIII of normal FX and FX Friuli genes. The mutation results in coding for serine (TCC) instead of praline (CCC) at position 343 in the FX Friuli molecule.

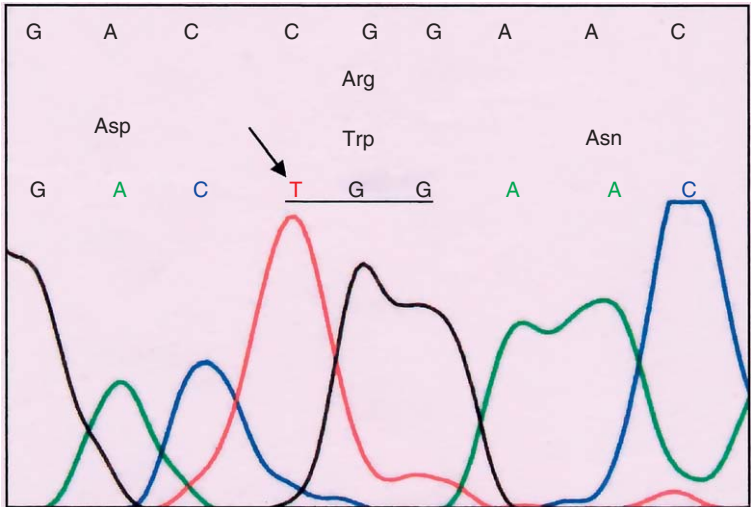


Figure 14.10 Sense DNA sequence of Exon VIII of FX Padua 3 gene resulting in the replacement of Arg with Trp at position 251.

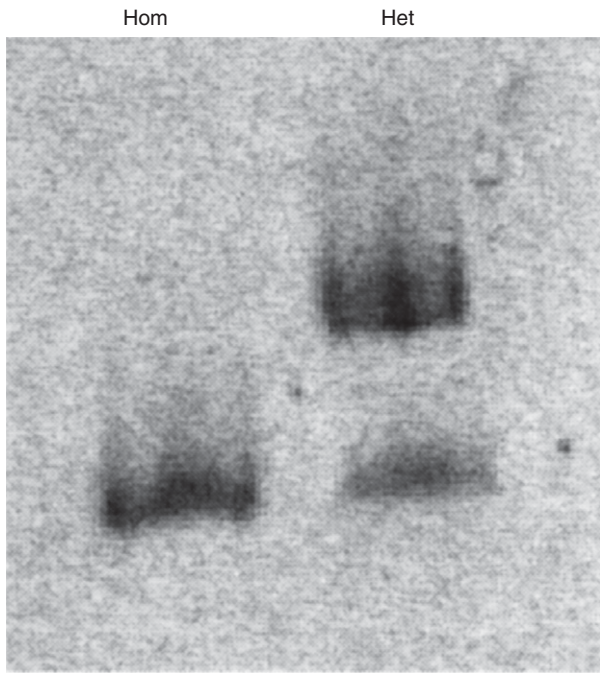


Figure 14.11 Conformation sensitive gel electrophoresis (CSGE) of PCR amplified Exon 8 in a Factor X abnormality (Arg251Trp, or Padua). This is a useful screening procedure for clotting factor abnormalities with normal or near normal antigen in their plasmas. Hom., homoduplex; Het., heteroduplex.

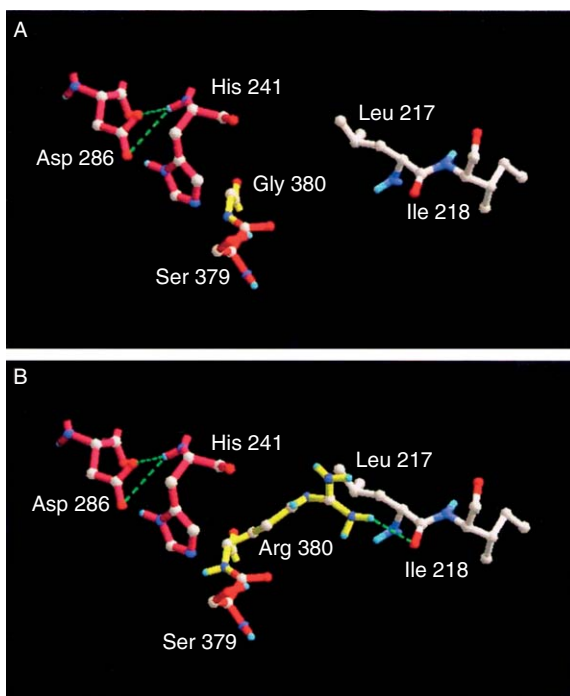


Figure 14.12 Putative tridimensional structure of normal FX and of FX Padua 3 characterized by the Gly380Arg mutation. Catalytic residues are shown in red; site of mutation in yellow; broken lines indicate hydrogen bonds.

F. Prognosis

Prognosis varies with the severity of defects. The limited number of cases described has not allowed the gathering of sure information on survival. Fatal brain hemorrhage is an important cause of death in early age. Levels of about 10% of normal seem sufficient to assure a decent life, providing no surgery, trauma, pregnancy, or invasive diagnostic procedures are needed. These levels may even afford some antithrombotic protection. In fact, one of the patients of the FX Friuli is alive and fairly well at the age of 103 ([Girolami *et al.*, 2005](#)). This is the only study of prognosis available, but it refers only to a specific abnormality (FX Friuli) in which homozygotes have FX activity levels around 6–8% of normal. It is also worth noting that in another of these patients who died at the age of 72 for a carcinoma of the pancreas, arteries were found, at autopsy, smooth for the age ([Girolami *et al.*, 1975a](#)).

G. Therapy

Plasma or PC concentrates are the available tools. No FX concentrate is available. PCC are usually titrated in FIX activity, and therefore their content in FX may vary. The relatively long half-life allows spaced

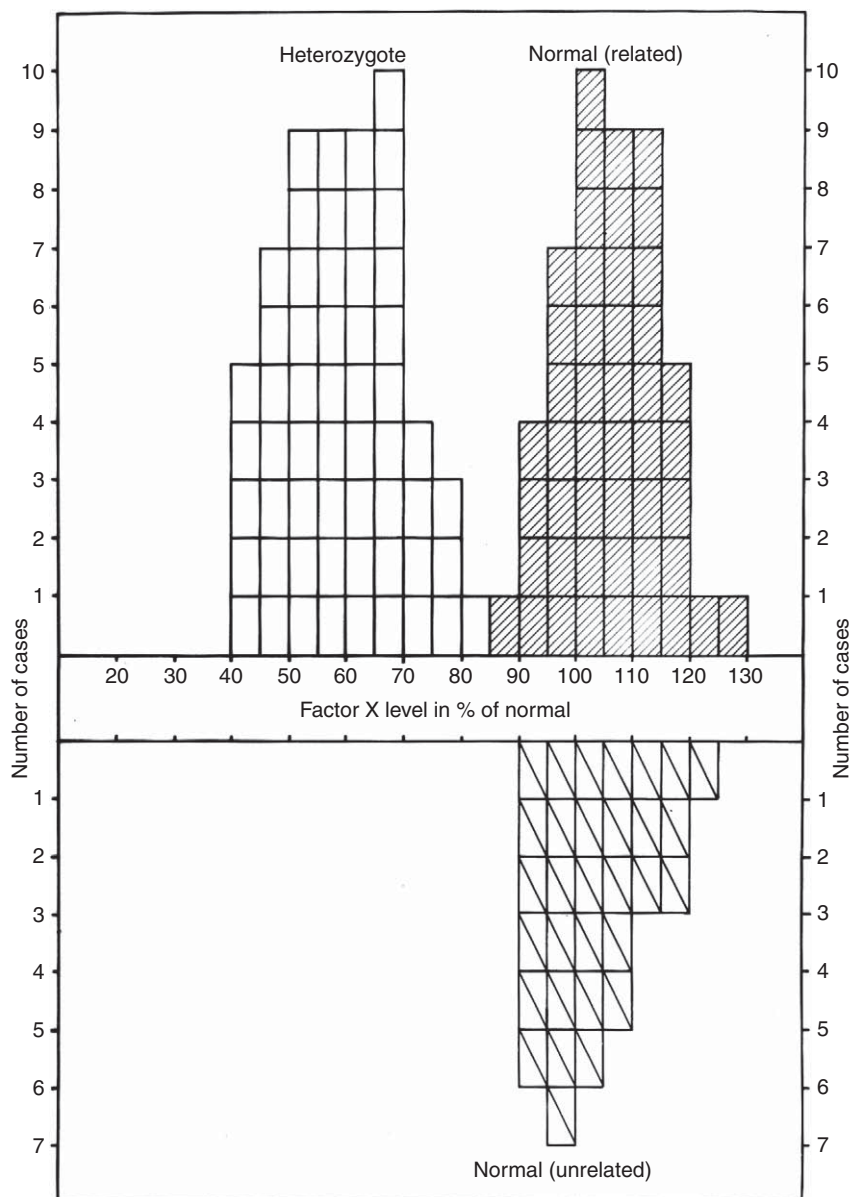


Figure 14.13 Factor X (FX) levels as determined by the extrinsic system in 57 heterozygotes for FX Friuli disorder and in 77 normal subjects. The normal subjects were both related and unrelated to the homozygote patients. The figure entered is the mean of at least two determinations carried out on different occasions. The overlay between affected and normal appears minimal, if any.

administrations. Safe hemostatic levels are at least around 40–50% of normal in case of surgery, trauma, and delivery. This is based on the observation that heterozygotes who have similar or slightly higher levels, only rarely bleed spontaneously (Figs. 14.14 and 14.15).

However, even heterozygotes may occasionally need transfusional support in cases of major surgical procedures.

Spontaneous bleeding in homozygotes or other patients (compound heterozygotes) with FX levels of around 10% is uncommon. Prophylaxis has been carried out in a few severe cases with good results (Auerswald, 2006). A satisfactory reduction of bleeding manifestations can be achieved, especially in children, with the administration of PC concentrates containing also FX with the weekly or biweekly infusion of 10–20 units/kg body

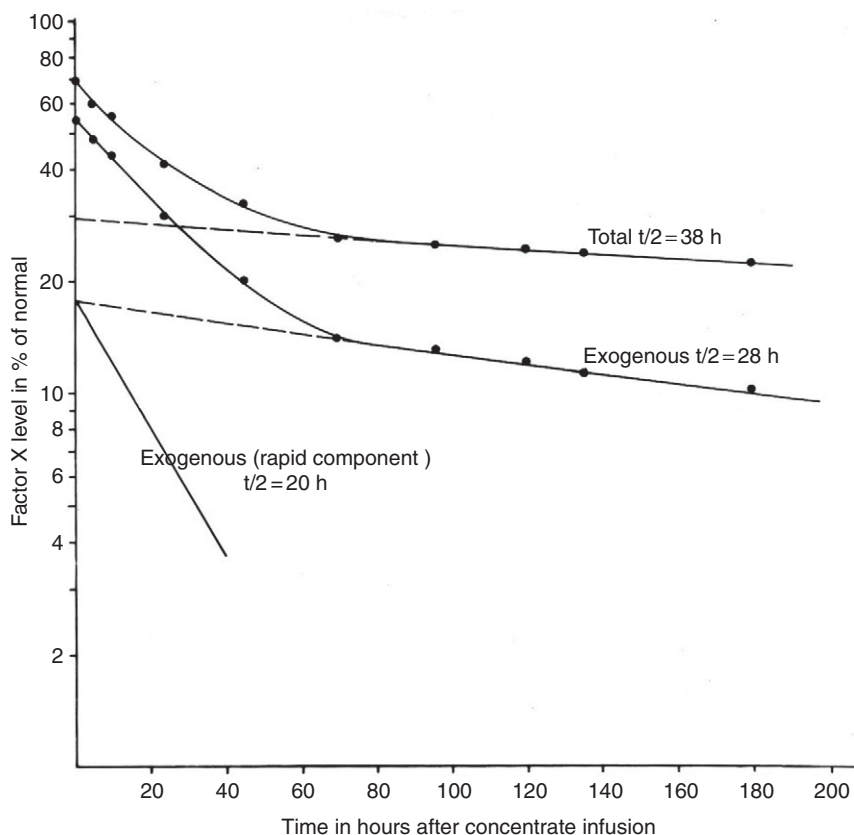


Figure 14.14 Factor X (FX) survival time after infusion of a prothrombin complex concentrate (2,000 units as titrated for FIX) in a patient with FX Friuli disorder (basal FX activity 5% of normal) before cholecystectomy. The survival time of exogenous compound is about 20 h. Surgical bleeding has been minimal.

weight (Auerswald, 2006). Oral contraceptives have been reported to be effective in reducing blood loss and in improving the anemia (Girolami *et al.*, 2006; Payne *et al.*, 2007). Pregnancies have been carried to term with adequate prophylaxis (Girolami *et al.*, 2006; Romagnolo *et al.*, 2004).

Antifibrinolytic agents have been used as adjunct measures. Orthotopic liver transplant has been carried out with success in at least one patient (McCarthy *et al.*, 1996). No attempts at genetic therapy have ever been reported.

H. Combined defects

FX deficiency has been rarely described in association with other clotting factors, namely, FVII, hemophilia A, and Factor XII deficiencies. The rarity of the disease may fully justify the scanty of reports.

The most important association seems to be that with FVII, as we have stated dealing with this factor. Two types of this association seem to exist, the first type due to the casual association of the two clotting factors that segregate independently in a given family. The second type seems to exist due to alterations of the long arm of chromosome 13 where both FX and FVII genes are located (13q34). The two genes are very close, and therefore deletions or other abnormalities may involve the two genes (Kasai *et al.*, 1989). Several cases have been described and patients present, usually, additional alterations, besides clotting defect (mental retardation, cardiac abnormalities, etc.). In this form, the hereditary pattern seems autosomal dominant.

Association with other clotting factors is rare and due to the casual combination of two, independently segregating defects.

VI. COMBINED CONGENITAL DEFICIENCY OF FII, FVII, FIX, FX

The congenital combined deficiency of all the PC factors including Protein C and Protein S is a rare but fascinating disease. It is often called VKCFD (vitamin K complex factor deficiency), and it has received considerable attention in recent years (Zhang and Ginsburg, 2006).

Only 28 cases belonging to 23 families can be gathered from the literature plus one so far unpublished from our files for a total of 29 cases.

The defect may therefore be considered as the rarest coagulation disorder.

The defect is due to mutation either in the γ -carboxylase or in the epoxide reductase systems. These enzymes play a critical role in the vitamin K metabolism or cycle.

A. Classification and prevalence

The defect is usually subdivided in two groups, namely, VKCFD Type I due to defective γ -carboxylase activity and VKCFD Type II. The Type II VKCFD is the result of a functional deficiency of vitamin K 2,3-epoxide reductase. This is the same enzyme that is involved in coumarin drugs therapy.

The first case was seen by [Newcomb *et al.* \(1956\)](#). Even though it was described before the separation of Factor X from Factor VII, a careful evaluation of the paper allows the conclusion that the propositus was indeed a case of congenital combined PC deficiency. The second case was the one presented by [McMillan and Roberts \(1966\)](#) and restudied by [Chung *et al.* \(1979\)](#).

At least 27 additional cases have been studied afterward ([Table 14.23](#)).

B. Biochemistry and function

The biochemical features of these factors have been dealt with while presenting the single factor deficiencies. They are all glycoproteins with important structural similarities.

γ -Glutaryl carboxylation of these proteins is needed for their activation. Such carboxylations allow for these proteins to achieve calcium-dependent structural changes necessary for binding to membrane phospholipids. The γ -carboxylation process also involves noncoagulation-related proteins, for example osteocalcin, and this may explain the occasional association in these patients with skeletal or other abnormalities.

Vitamin K is an important cofactor of the γ -carboxylation process ([Oldenburg *et al.*, 2007](#)). Vitamin K epoxide reductase (VKOR) is required to reconstitute the vitamin K hydroquinone form ([Fig. 14.16](#)).

Note that these hypocarboxylated or acarboxylated forms are present in both types of the condition. This is due to the fact that the defect in the epoxide reductase interferes with the carboxylation process by reducing the availability of reduced vitamin K (vitamin K hydroquinone).

C. Hereditary transmission and molecular biology

The hereditary transmission seems autosomal recessive, but no definite pattern has been established. Both homozygous and heterozygous have been occasionally described, but distinction is often not clear since parents of propositi show often a normal coagulation pattern. Both sexes are equally affected. There seems to be no sure ethnic preference, since the defect has been reported in several countries ([Bhattacharyya *et al.*, 2005](#); [Brenner *et al.*, 1998](#); [Chung *et al.*, 1979](#); [Fischer and Zweymuller, 1966](#); [Ghosh *et al.*, 1996](#); [Goldsmith *et al.*, 1982](#); [Johnson *et al.*, 1980](#); [Le Corvaisier-Pieto *et al.*,](#)

Table 14.23 Cases of combined prothrombin complex deficiency described in the literature

References	Age (years), sex	Consanguinity	Degree of defect	Correction by vitamin K	Molecular biology	Associated abnormality
Newcomb <i>et al.</i> (1956)	4, F	n.r.	Severe	Partial	n.r.	No
McMillan and Roberts (1966) and Chung <i>et al.</i> (1979)	0.4, F 15	No	Severe	Partial	n.r.	No
Fischer and Zweymuller (1966)	21, F	n.r.	Severe	Partial	n.r.	n.r.
Girolami <i>et al.</i> (1975) ^a	27, F	No	Moderate	Partial	n.r.	No
Mickelson and White (1979)	0.1,	n.r.	Severe	Partial	n.r.	No
Johnson <i>et al.</i> (1980)	2, M	No	Mild	No	n.r.	No
Goldsmith <i>et al.</i> (1982)	20, F	No	Mild	Yes	n.r.	No
Vicente <i>et al.</i> (1984)	1.2, F	No	Severe	No	n.r.	No
Ekelund <i>et al.</i> (1986)	14, M	n.r.	Severe	No	n.r.	No
Pauli <i>et al.</i> (1987)	7, M	No	Moderate	Partial	VKOR	Skeletal
Leonard (1988)	n.r.	n.r.	n.r.	Yes	n.r.	Yes
Le Corvaisier-Pieto <i>et al.</i> (1996)	40, M	No	Mild	n.r.	n.r.	Pseudoxanthoma elasticum
Boneh and Bar-Ziv (1996)	0.4, F	Yes	Moderate	Yes	n.r.	Skeletal
Ghosh <i>et al.</i> (1996)	4, M	No	Moderate	Partial	n.r.	Mental retardation
Brenner <i>et al.</i> (1998)						
Case 1	14	No (same family)	Moderate	Partial	Carboxylase	Skeletal

(continued)

Table 14.23 (continued)

References	Age (years), sex	Consanguinity	Degree of defect	Correction by vitamin K	Molecular biology	Associated abnormality
Case 2	7		Moderate	Partial	Id	No
Case 3	5		Moderate	Partial	Id	No
Case 4	4		Moderate	Partial	Id	No
Oldenburg <i>et al.</i> (2000)						
Case1	n.r.	Yes	Mild	Yes	VKOR	No
Case2	n.r.	No	Mild	Yes	VKOR	No
Spronk <i>et al.</i> (2000)	0.1, M	Yes	Moderate	Partial	Carboxylase	No
Mousallem <i>et al.</i> (2001)	0.2, n.r.	Yes	Severe	Partial	Carboxylase	No
McMahon and James (2001)	34, F	No	Mild	Partial	n.r.	No
Thomas and Stirling, 2003						
Case1	3	Yes	Mild	Partial	Carboxylase (deletion)	No
Case2	n.r.	Yes	Mild	Partial	Carboxylase	No
Puetz <i>et al.</i> (2004)	0.1, M	n.r.	Moderate	Partial	n.r.	Nervous system
Rost <i>et al.</i> (2004)	1, M	No	Mild	Partial	Carboxylase	Arteriosus ductus
Bhattacharyya <i>et al.</i> (2005)	n.r., M	Yes	Moderate	Partial	n.r.	No
Rost <i>et al.</i> (2006)	38, F	n.r.	Mild	Partial	Carboxylase	n.r.

^a Unpublished observation.

n.r., not reported or not available; VKOR, Vit K epoxide reductase.

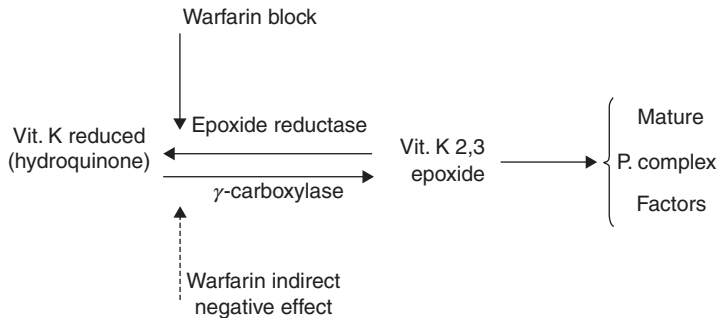


Figure 14.16 Schematic representation of vitamin K role in the formation of mature prothrombin complex factors. Vitamin K is needed for γ -carboxylase activity which is essential for the formation of active clotting proteins. Vitamin K epoxide is formed during the carboxylation process and has to be regenerated to active form (hydroquinone) by the vitamin K epoxide reductase (VKOR) system. This enzyme is blocked by Warfarin. Broken arrow indicates an indirect negative effect on γ -carboxylase activity due to decreased amounts of hydroquinone availability during Warfarin administration.

1996; Leonard, 1988; McMahon and James, 2001; McMillan and Roberts, 1966; Mickelson and White, 1979; Mousallem *et al.*, 2001; Newcomb *et al.*, 1956; Pauli *et al.*, 1987; Perry, 2002; Reitsma *et al.*, 1988; Rost *et al.*, 2004, 2006; Spronk *et al.*, 2000; Thomas and Stirling, 2003; Vicente *et al.*, 1984). Consanguinity is present in about 50% of families in which this aspect was looked for and reported.

Unfortunately, only a few of the described cases have been investigated from a molecular point of view. The gene controlling γ -carboxylase is located on chromosome 2p12 and consists of 15 exons that encode a 758 aminoacid peptide.

At least five different mutations in the γ -carboxylase gene have been described (Table 14.24).

These mutations are responsible for decreased glutamate-substrate binding, stabilization, or vitamin K binding. In addition, a few cases due to functional deficiency of the multiprotein complex vitamin K 2,3-epoxide reductase have been reported. Genes on short arm of chromosome 16 have been implicated for the encoding of this enzyme, but the specific genotype-phenotype relation of these mutations has not been fully clarified.

D. Clinical picture

Clinical picture is variable. A first suspicion may arise in infancy, especially in those infants who receive vitamin K prophylaxis and continue to manifest easy bruising.

Both sexes are equally involved. Besides easy bruising, bleeding from umbilical stump, hematomas, hematuria, gastrointestinal bleeding, and

Table 14.24 Mutations and biochemical changes found in the patients who have been studied from a molecular point of view

References	Defect	Exon	Mutation or alteration	Genotype	Comment
Pauli <i>et al.</i> (1987)	VKO	/	↑ Level VKO	?	
Brenner <i>et al.</i> (1998)	Carbox.	9	Leu394Arg	Homozygous	
Oldenburg <i>et al.</i> (2000)	VKO	/	↑ Level VKO	?	Two families
Spronk <i>et al.</i> (2000)	Carbox.	11	Try501Ser	Homozygous	
Mousallem <i>et al.</i> (2001)	Carbox.	11	Tyr501Ser	Homozygous	
Thomas and Stirling (2003)	Carbox.	1	Deletion (1056–1069)	Homozygous	
Rost <i>et al.</i> (2004)	Carbox.	3, 11	Splice site (deletion); Arg485Pro	Comp. heterozygous	
Rost <i>et al.</i> (2006)	Carbox.	9, 11	His404Pro; Arg485Pro	Comp. heterozygous	

VKO, Vitamin K epoxide; Carbox., γ -carboxylase; Comp., compound.

menorrhagia has been reported. Brain hemorrhage has also been described. Hemarthroses have never been reported. The clinical picture is similar to that seen in single factor deficiency of the PC. Usually, it is less severe than in severe FII or FX deficiency. The severity of bleeding may vary from time to time without apparent reasons, and patients may have periods with only minor bleeding manifestations. Parents are usually asymptomatic.

There is a limited but variable response to vitamin K administration. Often the administration of vitamin K intravenously or orally has partially or even almost fully corrected the defect decreasing, at least temporarily, the bleeding tendency. Occasionally, patients may appear asymptomatic. Variable skeletal abnormalities, mental retardation, and pseudoxanthoma elasticum have occasionally been seen in the patients.

E. Diagnosis

Laboratory diagnosis may be difficult because of the variability of results during the observation period.

Anti-vitamin K drugs, liver failure, and malabsorption must be excluded. Circulating anticoagulant has also to be excluded. PT-derived tests and PTT

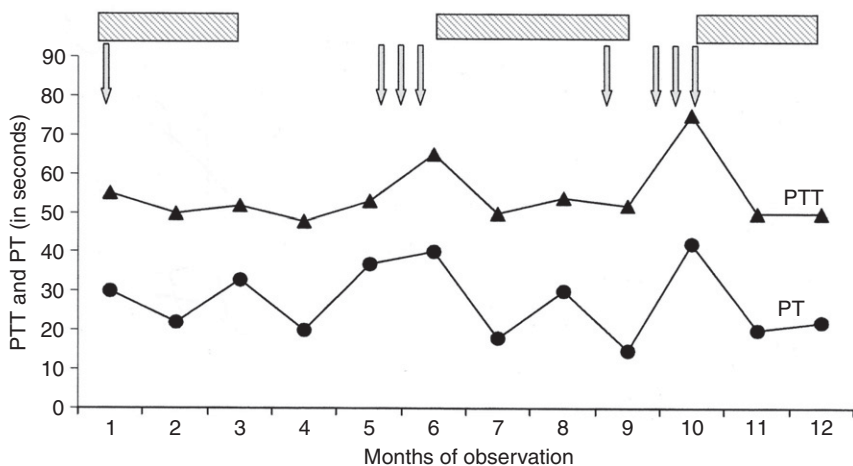


Figure 14.17 Behavior of PTT (normal 32–40 s) and PT (normal 13–15 s) during a 12-month observation period in a patient with combined deficiency of Factors II, VII, IX, and X. Arrows indicate bleeding episodes. Bars at the top of the figure indicate periods of vitamin K intake (usually oral). Partial and variable correction of clotting pattern is frequently observed in this condition.

are always, even though variably, prolonged. Thrombin time is normal. Cross-test with equal parts of normal plasmas are normal as expected in the absence of circulating anticoagulants. Protein C and Protein S, when measured, have been found decreased. Parents show a variable pattern that goes from normal to slightly or borderline levels of the four factors. The partial response to vitamin K administration should be of no surprise being typical for most cases with this defect (Fig. 14.17).

The factor levels vary during time, approaching sometimes, in less severe cases, the low normal range. Usually levels vary between 20% and 40% of normal. The levels of the four factors are often decreased to an unequal degree, due to the different half-lives possessed by them. Immunological assays always reveal high levels of antigens as compared with the clotting counterparts.

This is due to the presence of acarboxylated or hypocarboxylated forms in the circulation. Their presence is well demonstrated by a calcium-dependent bidimensional immunoelectrophoresis for FII or FX that shows the presence of two peaks or precipitates, since the acarboxylated forms are more anodic (Fig. 14.18). The same pattern may also be seen by means of a plain immunoelectrophoresis.

Short of molecular biology analysis, the finding of these decarboxylated forms in patients' plasma is the best clue for a correct diagnosis. Needless to say that coumarin drug and vitamin K deficiency from any cause have to be previously excluded. Elevated plasma or serum levels of vitamin K epoxide

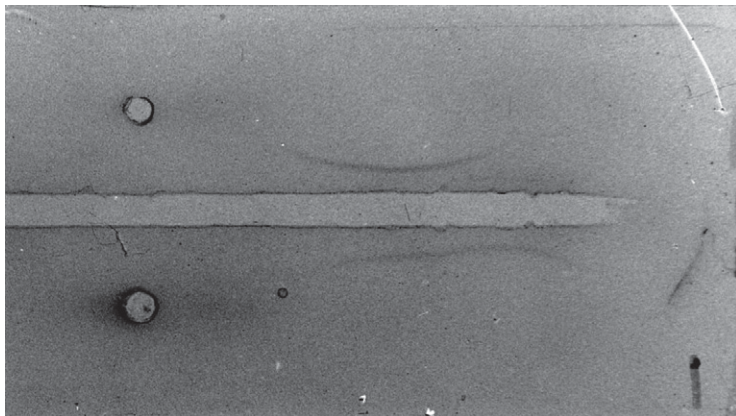


Figure 14.18 Immunoelectrophoresis of plasma of a patient with congenital combined deficiency of Factors II, VII, IX, and X. Upper run normal plasma, lower run patient plasma, anode to the right. In normal plasma only one precipitate is evident whereas two precipitates (normal and acarboxylated prothrombin) are seen in patient plasma.

(vitamin K 2,3) by chemical assays may sometimes be useful (Oldenburg *et al.*, 2000; Pauli *et al.*, 1987).

F. Prognosis and therapy

Prognosis varies with the severity of defect but has to be considered as guarded.

Because of the rarity of the disease, no sure data about survival are available. These patients represent a risk in case of surgical procedures.

Therapeutic measures are limited to continuous vitamin K oral administration. Vitamin K may also be given intravenously. Intramuscular administration is to be avoided for fear of hematomas. No data are available as to possible differences in response of the oral versus the intravenous administration.

In cases of need, plasma, PC, FVII, or FIX concentrates could be used. Response is good so that surgical procedures can be carried out.

Surgical procedures require levels of at least around 50%. The different half-lives of these factors (about 5 h for FVII and about 72 h for FII) may be responsible for discrepant posttransfusional levels. No data are available about liver transplant or gene therapy.

VII. DEFICIENCY OF PROTEIN Z

For the sake of completion, Protein Z deficiency should also be mentioned here, since a few sporadic observations or papers have suggested the possibility that this factor plays a role in bleeding.

Protein Z is a 62,000 single chain glycoprotein vitamin K dependent. It is synthesized in the liver under the coding of a gene located at chromosome 13q34, close to the genes encoding for FVII and FX. It has eight exons and a structure similar to these encoding for the other PC factors (Broze, 2006).

The possibility that Protein Z might be involved in a bleeding diathesis arose from the observations of decreased Protein Z levels in patients with a variable bleeding tendency, but without other clotting defects that the diathesis could be attributed to (Kemkes-Matthes and Matthes, 1995, 2001; Ravi *et al.*, 1998).

Other studies have not confirmed the findings and shown a great variability of Protein Z levels even in the normal population (Gamba *et al.*, 1998). Furthermore, it is interesting to know that no family has been described where both homozygotes (severe deficiency) and heterozygotes (mild deficiency) were present with an established hereditary pattern. Isolated cases with a severe deficiency have also never been reported.

Therefore, the role of protein Z in hemorrhagic disorders seems, at best, not proven and very unlikely.

The limited significance of anecdotal reports, the reported wide range of values in the normal population, the lack of satisfactory family pedigrees, and the vagaries of association studies are sufficient to explain the conflicting results. Therefore, Protein Z is still a clinically function orphan glycoprotein. Needless to say that future studies might clarify to pattern. For example, recent studies on the Protein Z-dependent protease inhibitory might clarify the pattern. In fact, since the activity of this inhibitor seems directed toward FXa, this could supply some support to a bleeding role of this protein at least an indirect one (Broze, 2006).

Protein Z has also been suggested to play both a prothrombotic and/or a protective role in patient with other thrombotic conditions (FV Leiden) on the basis of association studies. However even in this area, results are not univocal and therefore do not seem conclusive (Broze, 2006). This aspect is beyond the scope of this chapter and therefore is not discussed.

VIII. CONCLUSIONS

The discussion of the bleeding disorders of the PC is surely important and interesting. However, there is the suspicion that what we know at present is only a part of the picture.

It is remarkable that a vitamin K, widely spread in natural food, might be able to influence so much blood clotting.

Probably, the PC factors are somehow intertwined in their biological functions, the best example of homeostasis. Some factors protect from

bleeding, others protect from thrombosis and some, like Protein Z, probably have not found their role yet.

When coumarin drugs are given to a patient, both types of factors are decreased, but, clinically, the protection against thrombosis seems to prevail. However, thrombotic manifestation may occur even in fully anticoagulated patients. Does this mean that, in this setting, Protein C and Protein S deficiency play a role? We do not know.

Does Protein Z play a role? What are the clinically relevant relations between Protein Z, P2-dependent protease inhibitor and FXa?

A role by Proteins C and S is likely, since a quick anticoagulation may cause diffuse ischemic small vessel occlusions with secondary tissue necrosis, namely, “purpura fulminans” in patients severely deficient of Protein C or Protein S.

Is there a regulatory protein responsible for the balance of the two different groups of factors? How do Protein C and Protein S react in the presence of elevated levels of FII or FVII? We just do not know.

The different hereditary pattern shown by this group of factors is fascinating. The fact that only one of them is X-linked, whereas the other five are autosomal remains to be clarified. Does it have a phylogenetic significance?

Which of the two first? The sharp difference in biological half-lives shown by these factors, ranging from a few hours (FVII and Protein C) and a few days (FII), is also still lacking a proper explanation. Again, does it have a phylogenetic significance?

Altogether, these disorders probably account for only 15–20% of all congenital bleeding disorders (Acharya *et al.*, 2004; Blanchette *et al.*, 1991; Bolton-Maggs and Hill, 1995; Girolami *et al.*, 1985a), hemophilia A, von Willebrand disease, and FXI deficiency accounting for the remaining 80–85%. However, the historical importance of this group of disease is clear.

Their discovery has allowed the construction of an acceptable framework for the understanding of blood coagulation. It is also worthy of mention the fact that the first CRMpos variant ever discovered involved FIX (hemophilia B), followed by FII and FX and, finally, by FVII.

The study of these deficiencies was also instrumental in defining coagulation tests that then became routine, for example, in the follow-up of anticoagulated patients.

Probably the study of patients with these rare coagulation disorders during the past 40 years has also been rewarding at the personal level. On the basis of this experience, we surmise that the contribution of PC factors to blood coagulation is surely not exhausted yet. There is also a recent widespread interest in the extrac clotting activity of vitamin K. This followed the observation that carboxylase is present in several tissues. The relation existing between hepatic carboxylase and other tissue carboxylase is

still poorly understood. The study of the relations existing between the vitamin K-dependent clotting factors with the other vitamin K-dependent proteins such as osteocalcin and matrix-Gla protein (MGP) may supply further interesting results (Cranenburg *et al.*, 2007). A final comment on the overall results of the molecular biology investigations seems indicated. On the one hand, these techniques have allowed important advances (e.g., the classification of the pathological basis of combined FII, FVII, FIX, and FX deficiency); on the other, they have failed so far to supply definitive answers to the genotype–phenotype relation and/or to improve the therapeutic management of these disorders. The fact that identical mutations may, sometimes, give origin to different phenotypes (e.g., some of those underlying FVII deficiency) or that different mutations in different exons may cause the same phenotype (e.g., those causing hemophilia Bm) remains to be clarified. In addition, it has not been possible so far to indicate with certainty. The mutations, save for some deletions, associated with CRMpos or CRMneg forms. Refined studies of the purified and/or expressed protein will surely contribute to the understanding and explanation of these discrepancies.

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ROLE OF GROWTH ARREST-SPECIFIC GENE 6 IN DIABETIC NEPHROPATHY

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Abstract

Nephropathy is one of the most common and severe complications of diabetes mellitus. The mechanism of diabetic nephropathy, however, remains incompletely understood. To elucidate the mechanism of diabetic nephropathy, we focus on the role of a vitamin K-dependent growth factor, growth arrest-specific gene 6 (Gas6), and its receptor Axl in the pathogenesis of diabetic nephropathy. We used streptozotocin (STZ)-induced diabetic rats and mice as a model of diabetic nephropathy and examined the role of Gas6 and Axl in the development of diabetic nephropathy. We also studied signaling mechanisms involved in mesangial hypertrophy characteristic of the early phase of diabetic nephropathy *in vitro*. After 12 weeks of STZ injection, the glomerular expression of Gas6 and Axl was increased along with the phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1. Administration of warfarin, which inactivates Gas6, inhibited mesangial and glomerular hypertrophy and the increase in albuminuria in STZ-rats. Warfarin treatment also inhibited the phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1. To demonstrate the specific role of Gas6, we showed that these findings were recapitulated in STZ-induced Gas6-knockout mice and confirmed the role of Gas6 in the development of diabetic nephropathy *in vivo*.

In vitro stimulation of mesangial cells with Gas6 resulted in mesangial cell hypertrophy. Stimulation of the cells with 25 mmol/l of glucose increased the expression of Gas6/Axl and mesangial cell size compared with that with 5.6 mmol/l of glucose. LY294002 and rapamycin blocked Gas6-induced activation of the Akt/mTOR pathway and mesangial hypertrophy.

Thus, we have found a novel mechanism of glomerular hypertrophy through the Gas6/Axl-mediated pathway in the development of diabetic nephropathy, where the Akt/mTOR pathway is a key signaling cascade in Gas6-mediated mesangial and glomerular hypertrophy. Inhibition of the Gas6/Axl pathway in diabetic patients might be beneficial to slow down the progression of diabetic nephropathy. © 2008 Elsevier Inc.

I. INTRODUCTION

Diabetes is the most common cause of end-stage renal disease in many countries. Approximately 30% of type 1 diabetic patients suffer from diabetic nephropathy (Bojestig *et al.*, 1994; Krolewski *et al.*, 1996). Therefore, tremendous efforts have been made to elucidate the molecular mechanism of diabetic nephropathy to develop an effective treatment. The feature characteristic of diabetic nephropathy is persistent albuminuria and mesangial expansion followed by glomerulosclerosis and a decline in renal function. The development of glomerulosclerosis is preceded by the early hypertrophic processes in the glomerular compartment (Ziyadeh, 1993). Since it is important to regulate the early stage of the disease process, the role of growth factors and cytokines has been studied to determine their role in mesangial expansion and hypertrophy (Wolf, 2000). Transforming growth

factor- $\beta 1$ (TGF- $\beta 1$) and angiotensin II are found to be implicated in the development of diabetic nephropathy (Lehmann and Schleicher, 2000). Angiotensin-converting enzyme inhibitors can decrease the risk of developing diabetic nephropathy by 12.5% in type 2 diabetic patients (Ravid *et al.*, 1998) and losartan (angiotensin II type 1 receptor blocker) treatment resulted in significant improvement in renal outcomes in patients with type 2 diabetes and nephropathy (Brenner *et al.*, 2001), indicating the role of the renin-angiotensin system in the development of diabetic nephropathy. However, these treatments are not sufficient to cure diabetic nephropathy. Therefore, additional pathogenetic mechanisms are extensively investigated to help design novel therapies for patients with diabetic nephropathy.

Among other potential growth factors for glomerular cells, we have investigated the role of growth arrest-specific gene 6 (Gas6) in the pathogenesis of kidney disease. Gas6, cloned from serum-starved fibroblasts (Manfioletti *et al.*, 1993), is posttranslationally activated by γ -carboxylation of glutamate residues at its N-terminus in the presence of vitamin K and inhibited by the anticoagulant warfarin (Nakano *et al.*, 1995, 1997). We have found that Gas6 is an autocrine growth factor for mesangial cells, and that Gas6 and its receptor Axl play a critical role in the development of glomerulonephritis in a mesangial proliferative model of glomerulonephritis (Yanagita *et al.*, 2001, 2002). We, therefore, continued our work to determine the role of Gas6 and Axl in the development of diabetic nephropathy. We also studied the signaling mechanisms involved in mesangial cell hypertrophy and found that Gas6 induces mesangial cell hypertrophy by activating the Akt-mammalian target of rapamycin (mTOR) pathway in the pathogenesis of diabetic nephropathy. In this chapter, we show our *in vivo* and *in vitro* work demonstrating a crucial role of Gas6 and Axl in the development of diabetic nephropathy.

II. ROLE OF GAS6 IN STZ-INDUCED DIABETIC RATS

A. Expression of Gas6 and Axl in STZ-induced diabetic rats

To examine the role of Gas6/Axl in the early phase of diabetic nephropathy *in vivo*, we used streptozotocin (STZ)-induced diabetic rats. We analyzed the glomerulus after 12 weeks of STZ injection and found that expression of both Gas6 and Axl was significantly increased in the STZ-induced diabetic rats and that Gas6 and Axl were mostly localized in endothelial and mesangial cells (Fig. 15.1). We also isolated glomeruli from the rats and confirmed increased glomerular expression of Gas6 and Axl by Western blotting.

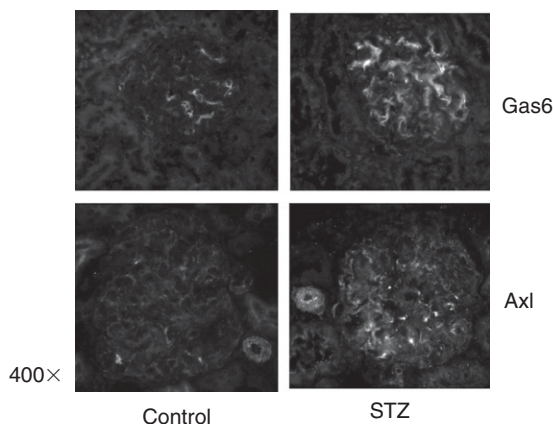


Figure 15.1 Expression of Gas6 and Axl in STZ-induced diabetic rat kidney. A single intravenous injection of 55 mg/kg of STZ was given on day 1 to make diabetic rats. After 12 weeks kidney tissues from each animal were snap frozen in cold acetone in OCT compound. Cryostat sections (4 μ m) were stained using indirect immunofluorescence procedure with anti-Gas6 or anti-Axl antibody. Original magnification, 400 $\times$. Abbreviations: Gas6, growth arrest-specific gene 6; STZ, streptozotocin (reproduced from Nagai *et al.*, *J. Biol. Chem.*, 2003).

B. Warfarin treatment inhibits induced expression of Axl

We next examined whether inhibiting the function of Gas6 can ameliorate this experimental diabetic nephropathy. We treated STZ-rats with warfarin to block the function of Gas6. Plasma concentrations of warfarin in these rats were less than 1 μ M, which were significantly lower than the ordinary therapeutic concentrations as an anticoagulant. The body weight and kidney weight/body weight were not changed by warfarin treatment. Significant prolongation of prothrombin times, anemia, or bleeding tendency was not observed in all the rats during the whole period of warfarin treatment. In this experiment, warfarin treatment inhibited the induction of Axl in diabetic rats by Western blot analysis. Considering the fact that Gas6 can upregulate the expression of Axl in mesangial cells *in vitro* (Yanagita *et al.*, 1999), warfarin could inhibit the effect of Gas6 in mesangial cells in STZ-rats.

C. Warfarin shows a beneficial effect on mesangial and glomerular hypertrophy

Since glomerular hypertrophy is one of the earliest structural alterations in diabetic nephropathy, we measured mesangial cell and glomerular surface areas in diabetic rat kidney and examined the effect of warfarin on glomerular hypertrophy. After 12 weeks of STZ injection, both mesangial cell and

glomerular areas were significantly enlarged compared with control rats, and administration of warfarin prevented the increase of mesangial and glomerular areas (Fig. 15.2). Because accumulation of mesangial extracellular matrix components is also an early structural change in diabetic nephropathy, we also measured the periodic acid-Schiff methenamine (PAM)-positive area in both groups. However, there was no change in the PAM-positive area between control and diabetic groups, indicating that there is no glomerular sclerotic change after 12 weeks of STZ injection. The increase of extracellular matrix followed by mesangial cell hypertrophy is one of the major characteristics in diabetic nephropathy (Wolf and Ziyadeh, 1999). Ziyadeh also showed that the development of irreversible renal changes in diabetes mellitus, such as glomerulosclerosis, is always preceded by the early hypertrophic processes in the glomerular compartment (Ziyadeh, 1993). In our experiment, however, we could not find excessive accumulation of extracellular matrix in STZ-rats at 12 weeks, although other investigators have reported increased deposition of extracellular matrix in a later phase of nephropathy in STZ-rats (Makino *et al.*, 1992). Since our purpose in this study was to determine the role of Gas6 in the initial phase of diabetic nephropathy, we analyzed up to 12 weeks after STZ injection. The correlation between Gas6 and diabetic glomerulosclerosis should be evaluated by treating the rats with warfarin for a longer time or using another animal model.

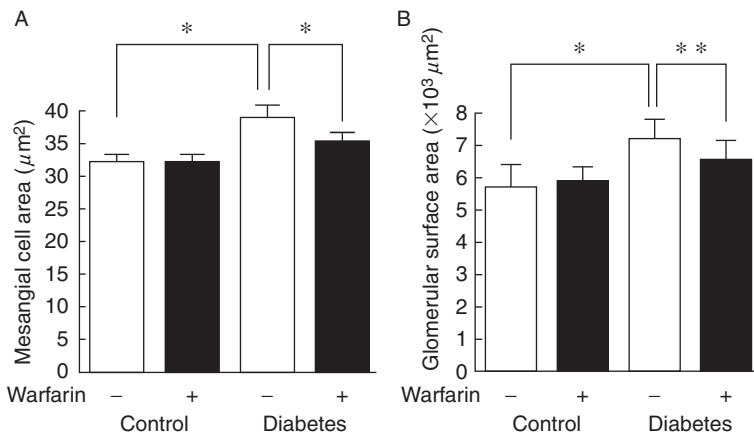


Figure 15.2 Effect of warfarin on mesangial (A) and glomerular hypertrophy (B). Rats were separated in control and diabetic groups with or without warfarin treatment. After 12 weeks the mesangial cell area was measured in a hematoxylin eosin staining section by Image-Pro Plus. For each animal, 50 mesangial cell areas were calculated. The glomerular surface area was determined using an image analyzer. Data are expressed as means $\pm$ S.D. ($n = 6$ in control group and $n = 10$ in diabetic group). * $p < 0.01$, ** $p < 0.05$ (reproduced from Nagai *et al.*, *J. Biol. Chem.*, 2003).

D. Warfarin treatment improves hyperfiltration and excretion of urinary albumin

In the early phase of diabetic nephropathy, glomerular filtration rate (GFR) is increased in most of diabetic patients. Therefore, we examined whether GFR is increased in STZ-rats and whether warfarin treatment can decrease GFR. After 12 weeks of STZ injection, GFR and urinary albumin excretion were significantly increased and the increased GFR and albuminuria were suppressed by warfarin treatment (Fig. 15.3). Warfarin has long been used as an anticoagulant to prevent thrombosis and embolism (Gallus *et al.*, 2000; Hostetter, 2001), and patients who are prescribed this agent are monitored by measuring prolongation of prothrombin times to achieve its anticoagulant effect. These patients have to be cared for the risk of bleeding (Booth and Mayer, 2000). However, the antihypertrophic effect of warfarin was achieved at serum concentrations of $0.7 \mu\text{M}$, which is significantly lower than the ordinary therapeutic concentrations as an anticoagulant ($4\text{--}5 \mu\text{M}$) (Schulman and Lindmarker, 2000). Our data also show that warfarin treatment ameliorated hyperfiltration and urinary albumin excretion in STZ-rats. Thus, hypertrophy and hyperfiltration might be causally linked (Stockelman *et al.*, 1998). It is conceivable, therefore, that blocking the Gas6/Axl pathway can improve the vicious cycle in diabetic nephropathy. Therefore, treating diabetic patients with warfarin to prevent the

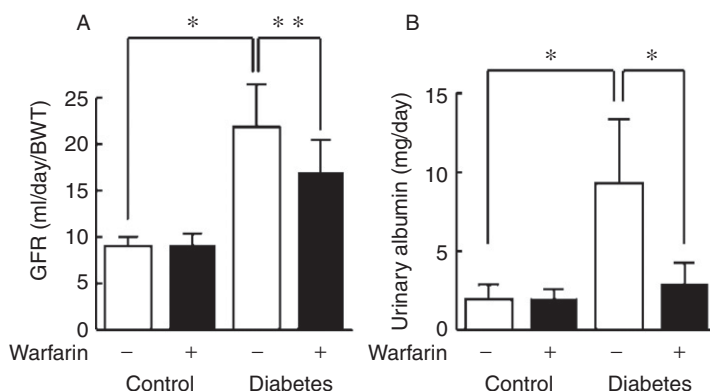


Figure 15.3 Effect of warfarin on hyperfiltration (A) and urinary albumin excretion (B). Urine volume was measured at 12 weeks in 24-h urine in each group of rats. Serum and urine creatinine concentrations were measured and GFR was calculated by an equation; $\text{GFR} = (C_u/C_p) \times V_u/\text{body weight}$, where C_p and C_u indicate serum and urine creatinine concentrations, respectively. Albumin concentrations in the urine were measured by Nephrot. Data are expressed as means $\pm$ S.D. ($n = 6$ in control group and $n = 10$ in diabetic group). * $p > 0.01$, ** $p > 0.05$. Abbreviations: BWT, body weight; GFR, glomerular filtration rate (reproduced from Nagai *et al.*, *J. Biol. Chem.*, 2003).

nephropathy would be one of the options for the treatment. Clinical studies should be performed to demonstrate the effect of warfarin to prevent the development of diabetic nephropathy in the future.

III. *IN VITRO* EFFECT OF GAS6 IN MESANGIAL CELLS

A. Gas6 induces mesangial cell hypertrophy *in vitro*

To investigate the molecular mechanism by which Gas6 causes glomerular hypertrophy in diabetic rats, we examined whether Gas6 can induce mesangial cell hypertrophy *in vitro*. We measured [^3H]-leucine incorporation in mouse mesangial cells as a marker of cellular hypertrophy after incubation with various concentrations of Gas6. Recombinant Gas6 increased incorporation of [^3H]-leucine dose-dependently, with a 1.5 fold-increase at maximum (Fig. 15.4). The same dose of Gla-defective Gas6, which is an inactive form of Gas6 without γ -carboxylation, did not affect [^3H]-leucine incorporation in mouse mesangial cells. The Gas6-mediated increase in [^3H]-leucine incorporation was almost the same as that of 1 ng/ml of TGF- β 1. To clarify whether mesangial cell hypertrophy is mediated

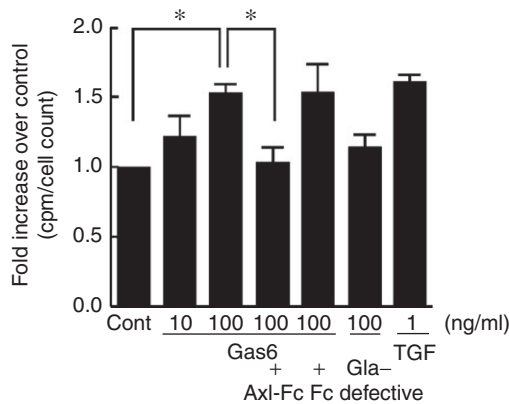


Figure 15.4 [^3H]-leucine incorporation in mouse mesangial cells by Gas6. Mesangial cells were plated at 1.5×10^4 cells/well in 24-well dishes. After 48 h, cells were serum-starved in DMEM containing 0.5% BSA for 48 h. Then the medium was replaced with the fresh starving medium including various concentrations of agonist, or left untreated. After 18 h, cells were labeled with [^3H]-leucine (2 $\mu\text{Ci}/\text{ml}$) for 6 h and the incorporation of [^3H]-leucine into acid-precipitable materials was determined. The data were normalized by dividing incorporated counts with cell number and showed as fold increases over control. Values expressed are the means $\pm$ S.D. of six independent experiments. $*p > 0.01$. Abbreviations: Gas6, growth arrest-specific gene 6; TGF, transforming growth factor (reproduced from Nagai *et al.*, *J. Biol. Chem.*, 2003).

specifically through the Gas6–Axl pathway, we used the recombinant extracellular domain of Axl (Axl-Fc), which is a recombinant fusion protein of the extracellular domain of Axl and human Fc portion, as an inhibitor of the Gas6–Axl pathway. Addition of Axl-Fc inhibited the increased [^3H]-leucine incorporation by Gas6, suggesting that the effect of Gas6 on hypertrophy is specific for the Gas6–Axl interaction. Thus, Gas6 has been added to the list of growth factors involved in mesangial cell hypertrophy.



IV. ROLE OF THE AKT PATHWAY IN DIABETIC NEPHROPATHY

A. Phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1 and induction of p27 expression in diabetic rat glomeruli

To further elucidate the molecular mechanism involved in Gas6-mediated glomerular hypertrophy, we examined the expression of Akt and its downstream signaling molecules, p70 S6 kinase and 4E-BP-1, in diabetic glomeruli, and found that Akt, p70 S6 kinase, and 4E-BP-1 were induced and phosphorylated in rat glomerular lysates after 12 weeks of STZ injection (Fig. 15.5). Warfarin treatment of STZ-rats inhibited their induction and phosphorylation. These data suggest that Gas6 is involved in glomerular hypertrophy via the Akt/mTOR pathway *in vivo*. We also examined the expression of p27, an inhibitor of G1 phase cyclin-dependent kinase. Intriguingly, its induction completely paralleled the phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1, indicating that the Akt/mTOR pathway itself could play a role in glomerular hypertrophy *in vivo*. To examine whether Akt is activated in mesangial cells, immunohistochemical analysis was performed in STZ-rats. We found that phosphorylation of Akt was increased mainly in mesangial cells and that its localization was almost the same with that of Gas6 (Fig. 15.6). These data suggest that Gas6-mediated Akt/mTOR activation is responsible for glomerular hypertrophy in the early phase of diabetic nephropathy. Previous studies have shown that Akt regulates proliferation (Choudhury, 2001; Ghosh *et al.*, 2001) and survival (Hiromura *et al.*, 2002) of mesangial cells *in vitro*. Others have shown the importance of Akt in AngII- and endothelin-1-induced mesangial hypertrophy *in vitro* (Gorin *et al.*, 2001, 2003; Goruppi *et al.*, 2002). *In vivo*, Feliers *et al.* (2001) have demonstrated that the Akt/mTOR pathway is activated in renal cortex in diabetic db/db mice, a model of type 2 diabetic nephropathy. However, the role of the Akt/mTOR pathway especially in mesangial hypertrophy in diabetic renal disease was not investigated *in vivo* and no study has linked *in vitro* and *in vivo* data. This is the first demonstration to show the role of the Akt/mTOR pathway in diabetic nephropathy *in vivo* and to connect *in vivo* data to *in vitro* data shown in Section IV.B.

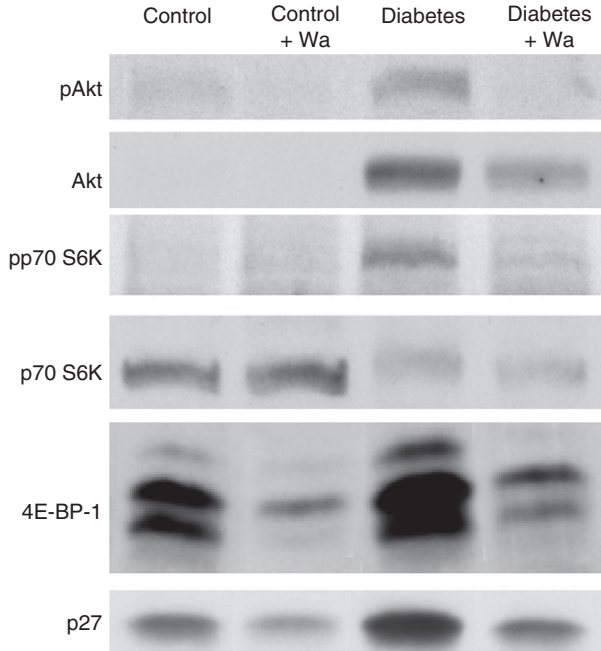


Figure 15.5 Activation of the Akt/mTOR pathway in diabetic rat glomeruli. After 12 weeks of STZ injection, isolated glomeruli were suspended in RIPA buffer. Sixty microgram of each sample was analyzed by Western blotting with the antibodies indicated. The bands immunoblotted with anti-p70 S6 kinase and anti-4E-BP-1 antibody increased in size when phosphorylated. Each lane represents a representative Western blot from each rat. Abbreviations: Wa, warfarin treatment; pAkt, phospho-Akt; pp70 S6 kinase, phospho-p70 S6 kinase; p4E-BP-1, phospho-4E-BP-1 (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

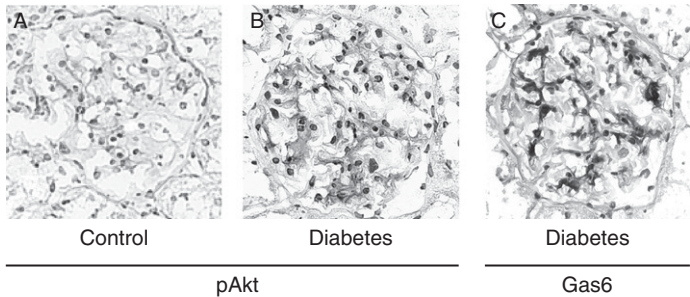


Figure 15.6 Expression and localization of phosphorylated Akt and Gas6 in STZ-rat glomeruli. Cryostat sections (4 μ m) were stained using indirect immunohistochemistry procedure with antiphospho-Akt (A, B) or anti-Gas6 (C) antibody. (A) is from control and (B) and (C) are from STZ-rats. The original magnification was 400 $\times$. Abbreviations: Gas6, growth arrest-specific gene 6; pAkt, phospho-Akt (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

B. Gas6 activates akt, p70 S6 kinase, and 4E-BP-1 *in vitro*

Because warfarin inhibited the hypertrophic effect of Gas6/Axl and activation of the Akt/mTOR pathway *in vivo*, we examined whether Gas6 can activate the Akt/mTOR pathway in mesangial cells *in vitro*. After stimulation of mesangial cells with the indicated concentrations of Gas6 for 30 min, phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1 was examined by Western blotting. As shown in Fig. 15.7, Gas6 increased phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1 in mesangial cells. The same dose of Gla-defective Gas6 did not affect their phosphorylation. Thus, Gas6 can activate the Akt/mTOR pathway *in vitro*.

C. LY294002 and rapamycin block Gas6-induced mesangial hypertrophy *in vitro*

We next examined whether LY294002, a PI3 kinase inhibitor, and rapamycin, an mTOR inhibitor, which are two representative inhibitors of the Akt/mTOR pathway, can affect mesangial hypertrophy and phosphorylation of the signaling molecules induced by Gas6. As expected both LY294002 and rapamycin inhibited Gas6-induced mesangial hypertrophy (Fig. 15.8). Under the same pretreatment protocol, cells were stimulated with 100 ng/ml of Gas6 for 30 min to examine their effect on the signaling cascade. Phosphorylation of Akt, p70 S6 kinase, and 4E-BP-1 was blocked by LY294002, while

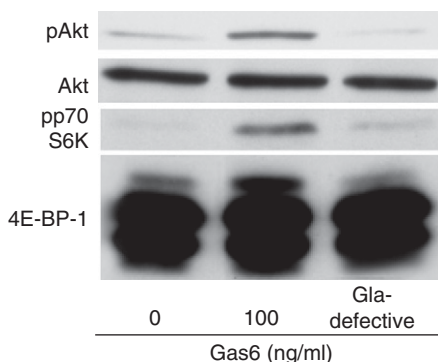


Figure 15.7 Gas6-mediated phosphorylation of the Akt/mTOR pathway in mesangial cells. Mesangial cells were plated at 1.5×10^5 cells/dish in 6-cm dishes. After 48 h, cells were serum-starved in DMEM containing 0.5% BSA for 48 h. Medium was changed to serum-free DMEM, and cells were incubated for another 2 h. The cells were then stimulated with the indicated concentrations of Gas6 or Gla-defective Gas6 for 30 min, and the cell lysates (20 μ g) were subjected to immunoblotting with the antibodies indicated. Abbreviations: Gas6, growth arrest-specific gene 6; pAkt, phospho-Akt; pp70 S6 kinase, phospho-p70 S6 kinase; p4E-BP-1, phospho-4E-BP-1 (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

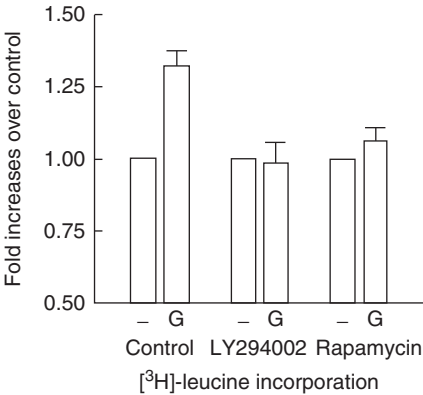


Figure 15.8 LY294002 and rapamycin can block Gas6-induced mesangial hypertrophy. Mesangial cells were serum-starved in DMEM containing 0.5% BSA for 48 h. After preincubation with 20 $\mu\text{mol/l}$ of LY294002 or 20 ng/ml of rapamycin for 1 h, the cells were stimulated with 100 ng/ml of Gas6. After 18 h, $[^3\text{H}]$ -leucine incorporation in the cells was determined as in Fig. 15.4. The values expressed are the means $\pm$ S.D. of four independent experiments (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

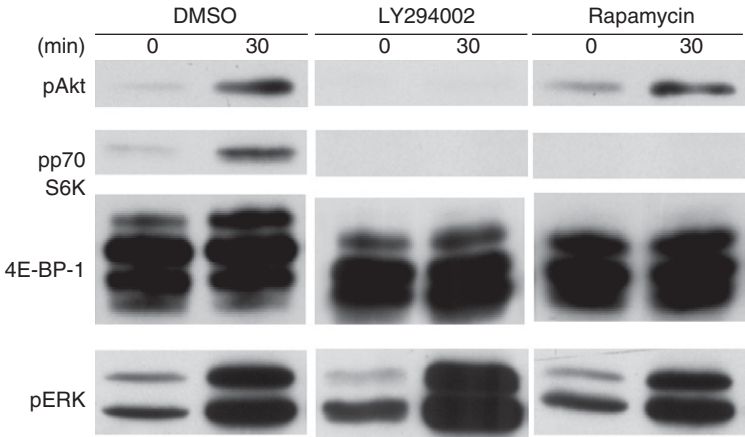


Figure 15.9 LY294002 and rapamycin can block Gas6-induced phosphorylation of the Akt/mTOR pathway. The cells were stimulated with 100 ng/ml of Gas6 for the indicated times, and their lysates (20 μg) were subjected to immunoblotting with the antibodies indicated. Abbreviations: pAkt, phospho-Akt; pp70 S6 kinase, phospho-p70 S6 kinase (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

phosphorylation of p70 S6 kinase and 4E-BP-1 was blocked by rapamycin as shown in Fig. 15.9. Lloberas *et al.* (2006) confirmed our finding *in vivo* by showing that blockade of the mTOR pathway by rapamycin actually prevents the progression of diabetic nephropathy in db/db mice.

To determine whether activation of Akt itself can induce mesangial hypertrophy, we transfected mesangial cells with myc-tagged cDNAs

expressing constitutive active, wild type (WT), or dominant negative mutants of Akt. Twelve hours after transfection, cells were serum-starved for 36 h. We then fixed the cells and stained them with anti-myc antibody. The size of cells expressing the myc epitope was then determined. The mesangial cell size transfected with constitutive active Akt was significantly greater than that with WT Akt ($2.64 \times 10^3 \pm 1.13 \times 10^3$ vs $2.39 \times 10^3 \pm 1.06 \times 10^3 \mu\text{m}^2$, $p < 0.05$) or dominant negative Akt ($2.31 \times 10^3 \pm 1.02 \times 10^3 \mu\text{m}^2$, $p > 0.01$). These data indicate that activation of Akt is sufficient to induce mesangial cell hypertrophy.

In this study we used rapamycin, an immunosuppressive agent, now available for organ transplantation in human. Our study suggests that rapamycin might be able to inhibit mesangial hypertrophy in diabetic nephropathy. Gooch *et al.* (2001) reported that IGF-1 can also induce mesangial hypertrophy via a calcineurin-dependent pathway, which indicates that another immunosuppressive agent, cyclosporine A, can prevent the mesangial hypertrophy in diabetes. There is another report that mycophenolate mofetil (MMF) ameliorated glomerular hypertrophy and glomerulosclerosis in experimental diabetic nephropathy (Utamura *et al.*, 2003). They suggest that this effect of MMF can be mediated through its anti-inflammatory effects. Although the direct effect of MMF on mesangial cells is not known, it is possible that MMF can also affect mesangial hypertrophy. Thus, these immunosuppressive drugs might prevent the progression of diabetic nephropathy by inhibiting mesangial hypertrophy in addition to their immunosuppressive and anti-inflammatory effects.

V. HIGH GLUCOSE INDUCES MESANGIAL HYPERTROPHY VIA GAS6/AXL *IN VITRO*

Why then is Gas6 increased in the diabetic state? To address this question, we examined the effect of high glucose on mesangial cell size by [^3H]-leucine incorporation *in vitro* and found that stimulation of the cells with high glucose increased the incorporation by 1.15-fold (Fig. 15.10A). This hypertrophic effect was inhibited by addition of Axl-Fc and abrogated in mesangial cells derived from Gas6-knockout mice (Fig. 15.10B), indicating a critical role of Gas6 in high glucose-induced mesangial hypertrophy.

The next step is to address the role of the Akt/mTOR pathway in this phenomenon. Therefore, we examined whether high glucose stimulation can induce the expression of Gas6/Axl and the activation of Akt/mTOR in mesangial cells *in vitro*. Stimulation of the cells with high glucose for 3 h resulted in an increased expression of Gas6 by 1.3-fold at mRNA levels compared with that with low glucose (Fig. 15.11A). Stimulation with high glucose for 36 h also significantly increased expression of Axl and phosphorylated Akt and its downstream molecules in mesangial cells derived

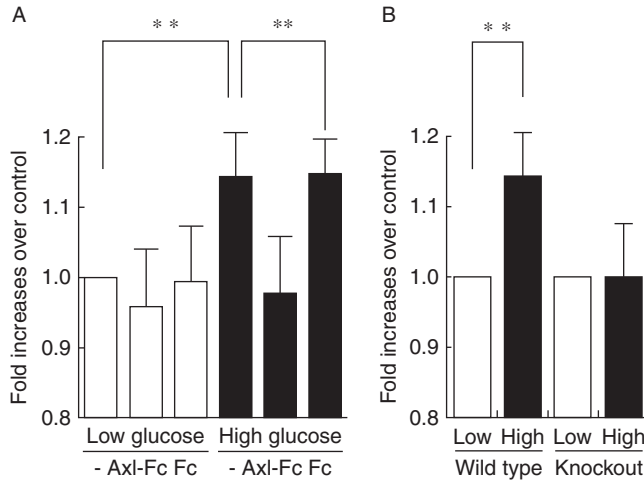


Figure 15.10 High glucose induces mesangial hypertrophy through Gas6 induction. (A) Mesangial cells were plated at 1.5×10^4 cells/well in 24-well dishes. After 48 h, cells were serum-starved in DMEM with either low (5.6 mmol/l) or high (25 mmol/l) glucose containing 0.5% BSA in the presence or absence of Axl-Fc (20 nmol/l). Human Fc portion (20 nmol/l) was used as control. After 24 h, cells were labeled with [^{3} H]-leucine (2 μ Ci/ml) for 12 h and incorporation of [^{3} H]-leucine and cell number were then determined. The data were normalized by dividing incorporated counts by the cell number and showed as fold increases over control. The values expressed are the means $\pm$ S.D. of four independent experiments. $**p > 0.05$. (B) Mesangial cells derived from wild type or Gas6-KO mice were stimulated with low or high glucose, and incorporation of [^{3} H]-leucine and cell number were then measured. The values expressed are the means $\pm$ S.D. of four independent experiments $**p > 0.05$ (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

from WT mice (Fig. 15.11B). These findings were not observed in mesangial cells derived from knockout mice. Thus, we have shown that Gas6 is upregulated in a high glucose state *in vivo* and *in vitro*. Meanwhile, p27 is one of the regulators of cell cycle and induces G1 phase cell cycle arrest (Wolf, 2000). p27 is upregulated in diabetic glomeruli and the molecular mechanism of mesangial hypertrophy in diabetic renal disease is, at least in part, explained by G1 phase cell cycle arrest. In our study, warfarin treatment of diabetic rats reduced the expression of p27 in diabetic rat glomeruli. Increased p27 expression in diabetic glomeruli might be explained by the induction of Gas6 in mesangial cells by high glucose state and that this might compose a vicious cycle to arrest DNA synthesis, followed by hypertrophy in mesangial cells. Although the upregulation of Gas6 by high glucose stimulation is relatively weak *in vitro*, this might be due to the weakness of stimulation by high glucose. Stimulation of mesangial cells for a longer period of time might result in more enhancement of Gas6 *in vivo* and further studies will be needed to explore the mechanism of Gas6 expression in diabetic state.

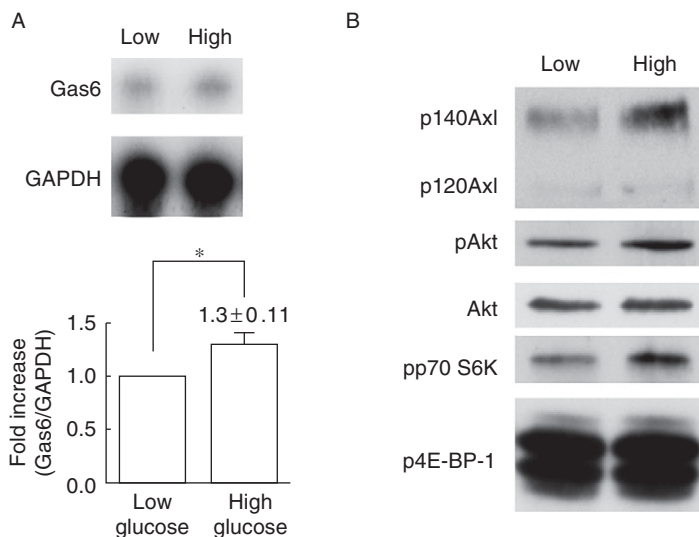


Figure 15.11 High glucose stimulates the Akt/mTOR pathway through Gas6/Axl-mediated pathway. (A) Mesangial cells were plated at 4.5×10^5 cells/dish in 10-cm dishes. After 48 h, the medium were replaced in DMEM with either low or high glucose containing 10% fetal calf serum. Three hours after the replacement, the cells were harvested and total RNAs were extracted and analyzed by ribonuclease protection assay. The protected bands for each RNA probe were evaluated by densitometric analysis. The values expressed are the means $\pm$ S.D. of three independent experiments. $*p > 0.01$. (B) Mesangial cells were treated as described in (A). After 36 h, cells were suspended in cell lysis buffer for the detection of p70 S6 kinase or RIPA buffer for the others. After centrifugation of the samples, the supernatants were used as total cell lysates. For the evaluation of p70 S6 kinase, the cell lysates were immunoprecipitated with anti-p70 S6 kinase antibody and immunoblotted with anti-p70 S6 kinase or phospho-p70 S6 kinase antibody. For the evaluation of the others, 20 μ g of each sample was applied to SDS-PAGE and immunoblotted with the antibody indicated. Representative data were shown from four independent experiments. Abbreviations: Gas6, growth arrest-specific gene 6; pAkt, phospho-Akt; pp70 S6 kinase, phospho-p70 S6 kinase. Low, low glucose; High, high glucose (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

VI. STUDY USING GAS6-KNOCKOUT MICE

A. STZ-treated Gas6-knockout mice showed less glomerular hypertrophy

To further confirm the specificity of Gas6 in diabetic nephropathy, we treated Gas6-deficient mice with STZ. As shown in Fig. 15.12, both mesangial and glomerular areas were significantly decreased in Gas6-deficient

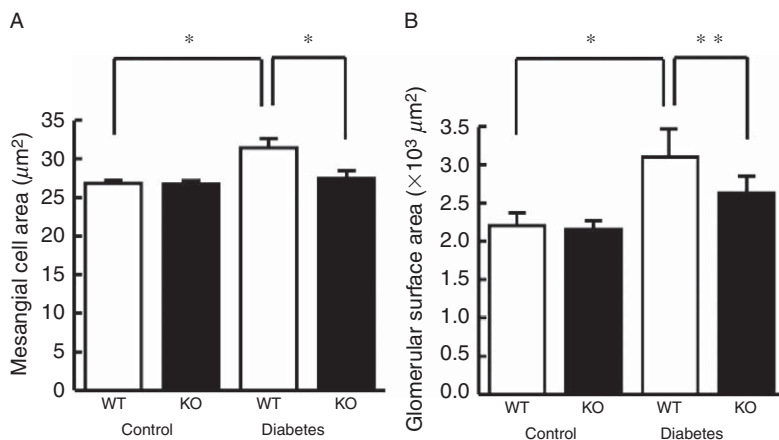


Figure 15.12 Effect of Gas6 deficiency on mesangial and glomerular hypertrophy in STZ-treated mice. Wild type (WT) and Gas6-knockout (KO) mice were divided into two groups: untreated (control) or STZ-treated (diabetes), respectively. Twelve weeks after STZ injection, mesangial cell and glomerular surface areas were measured. For each mouse, 50 mesangial cells and glomerular surface areas were calculated. The data are expressed as the means $\pm$ S.D. * $p > 0.01$, ** $p > 0.05$ (reproduced from Nagai *et al.*, *J. Biol. Chem.*, 2003).

mice compared with WT mice. Therefore, we can conclude that the effect of warfarin is mediated specifically through the inhibition of Gas6 and that Gas6 plays a critical role in diabetic nephropathy.

B. STZ-treated Gas6-knockout mice showed less activation of Akt in glomeruli

We also checked the phosphorylation of Akt in STZ-mice glomeruli. After 12 weeks of STZ injection, we stained the kidney section with antiphospho Akt antibody and measured the positive area. STZ-treated WT mice showed larger phosphorylated Akt-positive areas than did nontreated WT mice, while STZ-treated Gas6-knockout mice showed significantly less phosphorylated Akt-positive areas than did STZ-treated WT mice (Fig. 15.13), suggesting that Gas6-induced mesangial hypertrophy is specifically through the Akt/mTOR pathway *in vivo*.

VII. CONCLUSIONS

In this chapter, we have shown a novel mechanism of mesangial hypertrophy in diabetic nephropathy mediated by Gas6. Our study implies that Gas6 can be a novel growth factor that plays a crucial role in the development of the initial phase of diabetic nephropathy. Blocking this

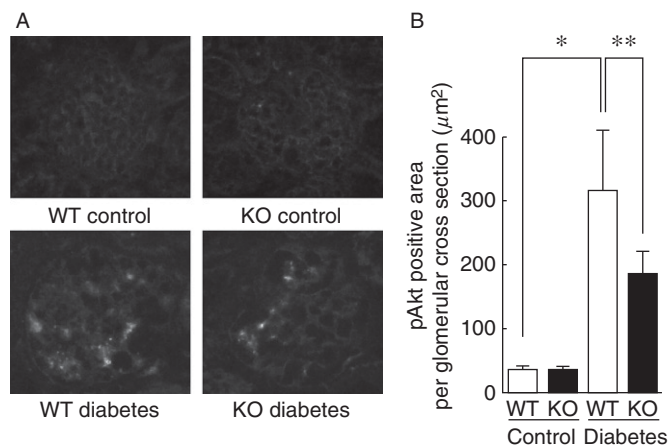


Figure 15.13 Effect of Gas6 deficiency on phosphorylated Akt and 4E-BP-1 expression in STZ-treated mice glomeruli. (A) After 12 weeks of STZ injection, WT and Gas6-KO mice were killed and the kidney tissues from each mouse were stained using indirect immunofluorescence procedure with antiphospho-Akt antibody. The original magnification was 400 $\times$. (B) Immunostained area was measured. The data are expressed as means $\pm$ S.D. ($n = 3$ in the control group and $n = 5$ in the diabetic group). * $p > 0.01$, ** $p > 0.05$. Abbreviations: pAkt, phospho-Akt; WT, wild type mice; KO, Gas6-knockout mice (reproduced from Nagai *et al.*, *Kidney Int.*, 2005).

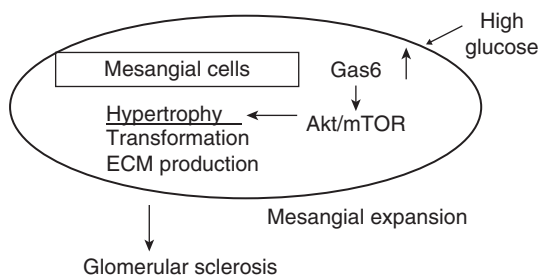


Figure 15.14 Schematic representation of Gas6-mediated mesangial hypertrophy in high glucose condition.

pathway would be beneficial to prevent the progression of nephropathy in diabetic patients.

As shown in Fig. 15.14, we propose a model of Gas6-mediated mesangial cell hypertrophy. It begins with high glucose stimulation of mesangial cells, followed by induction and activation of Gas6/Axl and the Akt/mTOR pathway, which leads to mesangial and glomerular hypertrophy. These data raise the intriguing possibility that Akt/mTOR activation might be a common main pathway in mesangial hypertrophy, and thus play a crucial role in the development of glomerular hypertrophy in the early phase of diabetic nephropathy.

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VITAMIN K AND BONE HEALTH IN ADULT HUMANS

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Abstract

Vitamin K is receiving more attention in relation to its role in bone metabolism. Vitamin K is a coenzyme for glutamate carboxylase, which mediates the conversion of glutamate to γ -carboxyglutamate (Gla). The γ -carboxylation of the Gla proteins is essential for the proteins to attract Ca^{2+} and to incorporate these into hydroxyapatite crystals. The best known of the three known bone-related Gla proteins is osteocalcin (OC). Even though the exact role of OC is not known,

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a number of studies have shown that vitamin K insufficiency or high levels of undercarboxylated osteocalcin (ucOC) is associated with an increase in the concentration of circulating ucOC. Furthermore, several studies have demonstrated that vitamin K insufficiency is associated with low bone mineral density (BMD) and increased fractures. Vitamin K supplementation, on the other hand, has been shown to improve the bone turnover profile and decrease the level of circulating ucOC. Dietary recommendations are based on saturation of the coagulation system, and in most countries the dietary intake is sufficient to obtain the amount recommended. In relation to bone, requirements might be higher.

The aim of this chapter is to give an overview of the importance of vitamin K in relation to bone health in adult humans and thereby in the prevention of osteoporosis. Furthermore, I will shortly discuss the interaction with vitamin D and the paradox in relation to warfarin treatment. © 2008 Elsevier Inc.

I. INTRODUCTION

A. Structure and function of vitamin K

Vitamin K is the generic name for a family of related compounds with similar γ -carboxylation activity. Vitamin K was discovered in 1929 by the Danish scientist Henrik Dam. Three compounds of vitamin K are known: Phylloquinone, or vitamin K₁, which is found mainly in green plants. Menaquinone, or vitamin K₂, which is a bacterial by-product and, therefore, mainly found in fermented products or in food from animal origin. Menaquinone comprises a family of molecules with an isoprenoid side chain of varying length from 1 to 14 repeats. When the isoprenoid side chain length is 0, it is called menadione, or vitamin K₃, which is a synthetic compound found only in supplements (Fig. 16.1).

For decades, it was believed that the sole function of vitamin K was in the coagulation cascade, but a number of other vitamin K-dependent proteins have been discovered. So far, it has amounted to at least 14 different vitamin K-dependent proteins. These comprise four procoagulants (factors VII, IX, X, and prothrombin) and three anticoagulant proteins (protein C, protein S, and protein Z), which are synthesized primarily in the liver. In addition, there are two proteins of bone and extracellular matrix [osteocalcin (OC) and matrix Gla protein], growth arrest-specific protein 6 (Gas6), which participate in a number of different processes (Berkner and Runge, 2004), as well as four membrane proteins with functions that have not yet been defined, the transmembrane Gla family, PRGP1, PRGP2, TMG3, and TMG4 (Kulman *et al.*, 1997, 2001). In addition to blood and bone, vitamin K-dependent proteins occur in dentin, renal stones, atherosclerotic plaques, semen, lung surfactant, neural tissue, and urine (Berkner

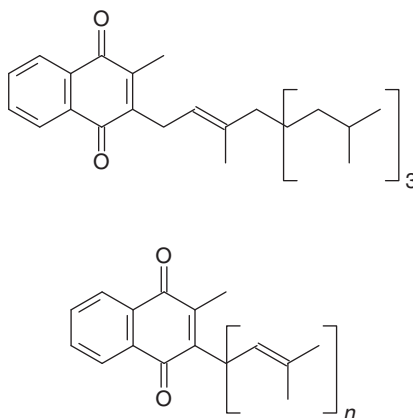


Figure 16.1 Structures of phyloquinone (vitamin K₁) and menaquinone (vitamin K₂).

and Runge, 2004). In all known cases, the Gla residues function as ligands for Ca^{2+} and are crucial for the biological activity of the proteins.

While γ -carboxylation of glutamic acid residues in vitamin K-dependent proteins is shared by vitamins K₁ and K₂, some vitamin K₂-specific, γ -carboxylation-unrelated functions have also been demonstrated. Menaquinone was recently shown to be a ligand for the steroid xenobiotic receptor (SXR) and thereby inducer of the mRNA synthesis for osteoblast biomarkers such as alkali phosphatase, osteoprotegerin, osteopontin, and matrix Gla protein (Tabb *et al.*, 2003).

B. Biosynthesis of Gla

Gla is biosynthesized as a posttranslational modification which is catalyzed by γ -glutamyl carboxylase, a resident enzyme of the endoplasmic reticulum. The polypeptide that is precursor for the modification comprises a single chain with an N-terminal signal peptide that is cleaved off prior to the synthesis of Gla. Binding of the substrate to the carboxylase is mediated by an adjacent propeptide of 18–28 amino acids. This binding activates the enzyme to γ -carboxylate the glutamyl residues located nearby (Berkner and Runge, 2004).

C. Vitamin K-dependent proteins in bone

Three vitamin K-dependent proteins have been identified in bones (Maillard *et al.*, 1992; Price and Williamson, 1985; Price *et al.*, 1976). The most abundant noncollagenous protein of bone, OC, is vitamin K dependent. It has also been called bone γ -carboxyglutamic acid (Gla) protein (BGP).

It is a small Ca^{2+} -binding protein that contains three Gla residues and it is indigenous to the organic matrix of bone, dentin, and possibly other mineralized tissues (Hauschka *et al.*, 1989). Matrix Gla protein is another Gla-containing protein associated with bone and cartilage. It contains five Gla residues and exhibits some peptide sequence homology to OC (Hauschka *et al.*, 1989). The Gla residues are Ca-binding groups, which are essential for the biological activity of the proteins; consequently, undercarboxylated proteins have low affinity for Ca and low biological activity (Vermeer *et al.*, 1998). The exact role of these proteins is not fully understood, but it is believed that OC is involved in regulation of the osteoblast and the growth of hydroxyapatite crystals (Iwamoto *et al.*, 2004), while matrix Gla protein is involved in bone formation and mineralization and as an inhibitor of calcification of arteries (Proudfoot and Shanahan, 2006). OC is synthesized by the osteoblast by a synthesis induced by $1,25(\text{OH})_2\text{D}_3$ (Hauschka *et al.*, 1989).

Ducy *et al.* (1996) showed that OC might be involved in limitations of bone formation without impairing bone resorption or mineralization. An animal study with knockout mice showed that knock out of the OC genes resulted in an increased bone formation without a concomitant increase in numbers or surface of osteoblasts. The numbers and surface of osteoclasts, however, were increased. When mutant and wild-type mice were ovariectomized, both developed an increase in bone-marrow area, which is a good indicator of osteoclast function. The mutant mice had, however, weaker bones than sham-operated mice, indicating that the mutant mice may develop a more severe osteoporosis than the wild-type mice after estrogen depletion. Furthermore, staining for mineralization showed no difference between wild-type and mutant mice (Ducy *et al.*, 1996). Another study showed that besides acting as an inhibitor of the osteoblast function, OC is also required for stimulation of bone mineral maturation (Boskey *et al.*, 1998). Knock out of the matrix Gla protein gene results in short stature, osteopenia, fractures, and accelerated calcification of arteries and cartilage (Luo *et al.*, 1997). This suggests that carboxylation of MGP may be important in bone formation and mineralization.

D. The first indications of a connection between vitamin K and bone health

In the mid-1970s, the first report on serious bone malformation in children born by mothers treated with vitamin K antagonists during the first trimester was published (Pettifor and Benson, 1975). Soon after, it was reported that patients who had sustained an acute hip fracture or suffered from a chronic spinal crush fracture had lower circulating phyloquinone in plasma compared with healthy controls (Hart *et al.*, 1984). This was later confirmed by Hodges *et al.* (1991, 1993), who showed that patients with osteoporotic

fractures of hip and spine had markedly decreased circulating levels of phylloquinone, menaquinone-7, and menaquinone-8. The question remains whether the decreased level of circulating vitamin K is the cause or a consequence of the fracture. The findings, however, have led to increased research in this area, and many studies have indicated that vitamin K is important for optimal bone health.

II. VITAMIN K INTAKE AND BONE HEALTH

A. Epidemiological studies

A number of epidemiological studies have shown a relationship between vitamin K intake and bone health. The two largest of these studies are the Framingham Heart Study and the Nurses Health Study (Booth *et al.*, 2000, 2003a; Feskanich *et al.*, 1999). Both of these studies have indicated that vitamin K intake, measured by food frequency questionnaire, correlates with hip fracture risk and bone mineral density (BMD). In the Nurses' Health study comprising 72,327 women aged 38–63 years, it was shown that an intake of $<109 \mu\text{g/day}$ of vitamin K was associated with a 30% higher risk of hip fractures than with higher vitamin K intakes (Feskanich *et al.*, 1999). Similar results were obtained by Booth *et al.* (2000) in 888 elderly men and women from the Framingham Heart Study. Booth *et al.* (2000) also showed that there was no association between vitamin K intake and BMD in the elderly men and women but found a significant lower BMD in the femoral neck in a group of younger women with low vitamin K intake ($70 \mu\text{g/day}$) compared to those with the highest intake ($309 \mu\text{g/day}$) (Booth *et al.*, 2003a). The lack of association between vitamin K intake and BMD first observed in men disappeared when values were adjusted for triglyceride and elevated undercarboxylated osteocalcin (%ucOC) (Booth *et al.*, 2003a) (Table 16.1).

1. Plasma phylloquinone concentrations and bone health

Plasma phylloquinone concentrations have also been shown to be associated with bone health in some studies (Booth *et al.*, 2004; McKeown *et al.*, 2002; Neogi *et al.*, 2006). Plasma phylloquinone concentration was shown to be inversely associated with %ucOC (McKeown *et al.*, 2002) and positively associated with BMD (Booth *et al.*, 2004). The associations between plasma phylloquinone and BMD were seen in both men and postmenopausal women not taking estrogen replacement, while no associations were found in premenopausal women or postmenopausal women taking estrogen replacement (Booth *et al.*, 2004). Among men, after adjustment for multiple covariates, low plasma phylloquinone concentrations and high %ucOC were associated with low BMD of the hip (femoral neck and trochanter).

Table 16.1 Observational cross-sectional studies (Studies on the relationship between vitamin K and bone health)

Study	Population	Outcome measurements	Results
(Feskanich <i>et al.</i> , 1999)	Nurses Health Study 72,327 women Age: 38–79 years	Vitamin K intake by FFQ Hip fracture risk	Hip fracture risk was 30% higher when vitamin K intake was $<109 \mu\text{g}/\text{day}$
(Booth <i>et al.</i> , 2000)	Framingham Heart Study 553 women, 335 men Age: 75.2 years	Vitamin K intake by FFQ Hip fracture risk and BMD	Hip fracture risk was significantly reduced (65%) in the group with the highest vitamin K intake ($254 \mu\text{g}/\text{day}$) compared to the group with the lowest intake ($56 \mu\text{g}/\text{day}$) No association between vitamin K intake and BMD
(McKeown <i>et al.</i> , 2002)	Framingham Heart Study 468 women, 369 men Age: 50– > 70 years	Vitamin K intake by FFQ Plasma phylloquinone and ucOC	Plasma phylloquinone was inversely associated with %ucOC There seems to be a threshold effect in plasma phylloquinone at intakes of $\sim 200 \mu\text{g}$ vitamin K /day
(Booth <i>et al.</i> , 2003a)	Framingham Heart Study 1,112 men, 1,479 women Age: 59 ± 9 years	Vitamin K intake by FFQ BMD	Women in the lowest quartile of vitamin K intake ($70 \mu\text{g}/\text{day}$) had significantly lower BMD at femoral neck and spine than women with the highest intake ($309 \mu\text{g}/\text{day}$) No association between BMD and vitamin K in men
(Booth <i>et al.</i> , 2004)	Framingham Heart Study 863 women, 741 men Age: 32–86 years	Plasma phylloquinone and %ucOC and BMD	Femoral neck BMD was significantly lower in men with low plasma phylloquinone adjusted for triglyceride and elevated % ucOC. Low plasma phylloquinone was associated with low spinal BMD in postmenopausal women not using hormonal replacement therapy

(Collins <i>et al.</i> , 2006)	97 healthy women Age: 50–75 years	Vitamin K intake by 14-day dietary recall, serum ucOC	34% had intakes below US recommendations and serum ucOC reflects intake
(Neogi <i>et al.</i> , 2006)	Framingham Heart Study 672 participants, 358 women Age: 65.6 years	Plasma phylloquinone and osteoarthritis in hand and knee	Low plasma phylloquinone concentrations were associated with increased prevalence of osteoarthritis in hand and knee
(Beavan <i>et al.</i> , 2004)	50 Gambian women aged 67 years + 11 aged 37 years 31 UK women aged 67 years + 11 aged 36 years 23 Chinese women aged 68 years + 11 aged 31 years	Plasma ucOC, plasma phylloquinone, apo E	Adjusted ucOC (adjusted for total OC) was lowest in China and highest in UK in postmenopausal women. Plasma phylloquinone was highest in Chinese and lowest in British women. Apo E4 frequency was as follows: Gambia: 33%; UK: 14%; and China 6%. A lower adjusted ucOC was associated with apo E2 genotype in UK and China
(Yan <i>et al.</i> , 2005)	85 Chinese men, 66.8 years 92 Chinese women, 64.4 years 66 UK men, 69 years 66 UK women, 67.9 years	Plasma phylloquinone, TCA, total OC, ucOC, apo E genotype, hip BMC	Plasma phylloquinone was generally higher and %ucOC was generally lower in Chinese than in British subjects. A higher plasma phylloquinone concentration was found in subjects with E3/4 + E4/4 than in those with either E2/3 or E3/3 in UK. A similar trend was seen in China. Subjects with E3/4 + E4/4 had a lower %ucOC than did those with E2/3 or E3/3 in China but not in UK

Abbreviations: BMD, bone mineral density; FFQ, food frequency questionnaire; ucOC, undercarboxylated osteocalcin; OC, Osteocalcin; TCA, triacylglycerol; BMC, bone mineral content.

With each 1 nmol/l of plasma phyloquinone, there was an increase of 0.007 g/cm² in BMD at the femoral neck. Among premenopausal women and postmenopausal women not using hormone replacement therapy (HRT), there were no associations between plasma phyloquinone concentration or %ucOC and BMD. Among postmenopausal women not using HRT, plasma phyloquinone concentrations were positively associated with BMD at the spine (Booth *et al.*, 2004).

The epidemiological studies indicate that a low intake of phyloquinone increases the risk of hip fractures. The association between phyloquinone intake and BMD was weak and probably also related to other factors, such as triglyceride concentration and %ucOC, while plasma phyloquinone concentration seems to be positively associated with BMD.

The use of epidemiological studies is limited and has been criticized for the potential confounding effect of overall poor nutritional status (Binkley and Suttie, 1995). Furthermore, vitamin K is primarily found in green vegetables which do also contain a number of other compounds that may have health benefits. As such, vitamin K might just be a marker for a certain lifestyle that has a beneficial effect on bone health. Therefore, well-controlled interventions studies were and are still needed to confirm the interaction between vitamin K and fracture risk.

B. Intervention studies

1. Dietary intervention and effect on risk markers

Most intervention studies have been performed over short time and have used biomarkers for bone formation and bone resorption as end points. In two such studies, 1 mg phyloquinone was given for 2 weeks to healthy pre- ($n = 100$) and postmenopausal ($n = 219$) volunteers, respectively, in the two studies (Binkley *et al.*, 2000; Knapen *et al.*, 1989). Bone formation and resorption markers were not affected by the phyloquinone treatment, except for urinary hydroxyproline and Ca, which decreased in a group of postmenopausal women categorized as fast Ca losers (Knapen *et al.*, 1989). γ -Carboxylation of OC increased in both studies. In contrast, Craciun *et al.* (1998) observed an increase in bone formation markers and a decrease in bone resorption markers in eight female long-distance runners (age: 20–44 years) given 10 mg phyloquinone for 30 days. These alterations were coherent with an increase in γ -carboxylation of OC and a decrease in urinary Ca excretion. This study, however, is difficult to compare with other studies as the subjects were elite runners having estrogen levels comparable to those of postmenopausal women and FSH and LH levels even lower. Furthermore, half the subjects used oral contraceptives, while the other half did not. The biochemical markers measured were very different from values found in control populations (Craciun *et al.*, 1998) (Table 16.2).

Table 16.2 Intervention studies

Study	Design	Dose	Duration	Outcome parameter	Results
(Knapen <i>et al.</i> , 1989)	50 postmenopausal women 50 premenopausal women	1 mg phylloquinone per day	2 weeks	Urine Ca, hydroxyproline, binding capacity of OC, and total OC	Vitamin K decrease urine Ca and hydroxyproline in postmenopausal women (fast losers). No effect was seen in premenopausal women
(Binkley <i>et al.</i> , 2000)	219 healthy subjects, Age: 18–30 years or >64 years	1 mg phylloquinone per day	2 weeks	Total OC, ucOC, NTx, BAP, and plasma phylloquinone	Plasma phylloquinone increased in all supplemented subjects from ~0.9 nmol/l to ~8.9 nmol/l. Mean %ucOC decreased from 7.6% to 3.4%
(Craciun <i>et al.</i> , 1998)	8 female long-distance runners Mean age 35 years	10 mg phylloquinone per day	30 days	ucOC, bone turnover markers, Urine Ca, and Gla	Vitamin K increased the bone formation markers (15–20%) and decreased the bone resorption markers (20–25%). Vitamin K also increased carboxylation of OC and decreased urine Ca excretion

(continued)

Table 16.2 (continued)

Study	Design	Dose	Duration	Outcome parameter	Results
(Schaafsma <i>et al.</i> , 2000)	23 postmenopausal women	80 μ g phylloquinone per day or 80 μ g phylloquinone + vitamin D per day	1 year		Vitamin K reduced the postmenopausal elevation in ucOC to premenopausal levels
(Booth <i>et al.</i> , 2001)	15 adults, aged 20–40 years	10 μ g phylloquinone per day 200 μ g phylloquinone per day	15 days 10 days	Bone formation and resorption markers	There was an increase and subsequent decrease in measures of bone formation and resorption after dietary phylloquinone restriction and repletion, respectively
(Booth <i>et al.</i> , 2003b)	21 women, aged 60–80 years	90 μ g 18 μ g/day 86 μ g/day 200 μ g/day 450 μ g/day All doses $\pm$ calcitriol	14 days 28 days 14 days 14 days 14 days	Plasma phylloquinone, urinary Gla, and PIVKA-II %ucOC	Depletion of vitamin K reduced γ -carboxylation of PIVKA-II in liver and OC in bone. Repletion caused a stepwise increase in γ -carboxylation of

(Braam <i>et al.</i> , 2003a)	155 postmenopausal women, aged 50– 60 years	MDK: 1 mg phylloquinone + vitamin D and min.mix MD: vitamin D + min. mix	3 years	BMD at femoral neck and spine	PIVKA-II in preference for γ -carboxylation of bone Gla proteins. The γ -carboxylation of PIVKA-II was restored to normal at intake of 200 $\mu\text{g}/\text{day}$ while no other markers of vitamin K status were restored at intakes of 450 $\mu\text{g}/\text{day}$ Reduced bone loss at femoral neck but not at lumbar spine with MDK compared to placebo. MDK better than MD

Abbreviations: BAP, bone alkaline phosphatase; BMD, bone mineral density; Gla, γ -carboxyglutamic acid; MDK, mineral vitamin D and vitamin K; MD, mineral and vitamin D; NTx, N-terminal crosslinked telopeptide of type I collagen; OC, Osteocalcin; PIVKA-II, protein induced by vitamin K absence; ucOC, undercarboxylated osteocalcin.

In depletion–repletion studies, it has been shown that with insufficient vitamin K intakes it is possible to induce changes in bone formation and resorption markers, which are restored to normal upon repletion with vitamin K in postmenopausal women (Booth *et al.*, 2003b). In young subjects, however, total OC and ucOC are changed upon depletion and repletion, while changes in resorption and formation markers are inconsistent or nonsignificant (Booth *et al.*, 2001).

A number of studies have shown that low intake of vitamin K is associated with a high amount of circulating ucOC and that supplementation with vitamin K can decrease the amount of circulating ucOC (Binkley *et al.*, 2000; Booth *et al.*, 2001, 2003b; Bügel *et al.*, 2007; Collins *et al.*, 2006; Craciun *et al.*, 1998; Knapen *et al.*, 1989; Schaafsma *et al.*, 2000). Booth *et al.* (2003b) showed that the level of ucOC is a better marker for vitamin K status than is protein induced by vitamin K absence (PIVKA-II). However, so far it is unclear what the physiological effects of a high amount of ucOC are; therefore, the ultimate effect of vitamin K on bone health would be a decrease in fracture risk.

2. Bone loss and fracture risk

A meta-analysis published in 2006 suggested that supplementation with either vitamin K₁ or menaquinone-4 reduces bone loss and decreases the incidents of fractures (Cockayne *et al.*, 2006). Of 352 potentially relevant articles, only 13, mainly Japanese studies, were included in the meta-analysis. Two of the studies used vitamin K₁ (Braam *et al.*, 2003a,b), while 10 of the remaining 11 trials used menaquinone-4 in doses of 45 mg/day. Furthermore, most of the studies were done in patients with secondary osteoporosis and/or in patients with a concomitant low intake of vitamin D and Ca.

Of the seven studies reported that measured fracture risk, three studies were with osteoporotic patients, while the remaining four studies used patients with Alzheimer's, Parkinson's disease, stroke, or kidney disease (Cockayne *et al.*, 2006). In all the studies reported, however, vitamin K intervention decreased the fracture risk (Cockayne *et al.*, 2006).

Only two studies have been performed in which the effect of vitamin K₁ on fracture risk was measured. Fracture risk was not affected by phylloquinone intake in perimenopausal women (Rejnmark *et al.*, 2006), while supplementation with a mixture of minerals, vitamin D, and vitamin K reduced bone loss in postmenopausal women ($n = 155$), when comparing with supplements of the same mixture of minerals and vitamin D, but without addition of vitamin K, or placebo (Braam *et al.*, 2003a).



III. REASONS FOR THE INCONSISTENT RESULTS

A. Differences between vitamins K₁ and K₂

It cannot be ruled out that there are differences in the effect of vitamins K₁ and K₂; therefore, more long-term studies are needed to ascertain that vitamin K₁ does have an effect on fracture risk. Furthermore, studies are lacking that compare the effects of vitamins K₁ and K₂ in order to rule out that the different effects seen may be because of ethnic differences (Beavan *et al.*, 2004; Yan *et al.*, 2005).

Besides the obvious possibility that vitamins K₁ and K₂ differ with respect to their action on bone metabolism and thereby also differ with respect to prevention of osteoporosis and bone fractures, a number of other factors might be important in order to explain the different results found in different studies, for example, absorption, metabolism, and excretion; dietary intake and source, as well as choice of biomarkers.

B. Vitamin K absorption, metabolism, and excretion

Phylloquinone is absorbed in the jejunum and ileum in a process that is dependent on the concomitant presence of dietary fat, as well as a normal flow of bile and pancreatic juice (Shearer *et al.*, 1970). Free phylloquinone is absorbed almost 100% (Shearer *et al.*, 1970), while phylloquinone from food is less well absorbed (Gijssbers *et al.*, 1996). The bacterial menaquinones synthesized by the gut microflora may also occur, as evidenced by the presence of bacterially derived menaquinone in the liver, particularly MK-10 and MK-11 (Thijssen and Drittij-Reijnders, 1996). Phylloquinone is absorbed into the lymph and transported to the liver in chylomicrons (Kohlmeier *et al.*, 1996). The availability of phylloquinone to bone is most likely influenced by apolipoprotein E (apo E) that acts as a ligand for cellular uptake from the lipoproteins. The apo E gene is polymorphic, with three common alleles resulting in six genotypes (E2/2, E2/3, E2/4, E3/3, E3/4, and E4/4) (Kohlmeier *et al.*, 1996). Apo E genotype has been linked to osteoporotic fracture risk (Cauley *et al.*, 1999; Kohlmeier *et al.*, 1998), to low BMD (Shiraki *et al.*, 1997), and to the lipoprotein-mediated transport of phylloquinone to bone (Kohlmeier *et al.*, 1997; Saupe *et al.*, 1993) although not all studies find associations between apo E genotype and BMD or fracture risk (Booth *et al.*, 2000; Harrington *et al.*, 2005). The chylomicron-remnant clearance is thought to be slowest in people with genotype E2/2 and fastest in those with genotype E4/4; clearance rates in those with genotype E3/3 lies in between and the heterozygous genotypes have intermediate clearance rates to the respective homozygous genotypes (Kohlmeier *et al.*, 1996).

Differences in genotype in different populations could result in different results. A few studies have been undertaken in which different populations were compared (Beavan *et al.*, 2004; Yan *et al.*, 2005). One study compared plasma ucOC, plasma phyloquinone, and apo E genotype frequency in post- and premenopausal women from Gambia ($n = 50 + 11$), UK ($n = 31 + 11$), and China ($n = 23 + 11$) and showed that adjusted ucOC was lowest in postmenopausal women from China and highest in postmenopausal UK women, and opposite for plasma phyloquinone. The apo E4 frequency was 33% in Gambian women, 14% in UK women, and 6% in Chinese women. A low ucOC was associated with apo E2 genotype in UK and Chinese women (Beavan *et al.*, 2004). When comparing elderly Chinese and British men ($n = 85$ and 66) and women ($n = 92$ and 66), it was found that plasma phyloquinone in general was higher and %ucOC lower in Chinese than in British subjects. In the British population, a higher plasma phyloquinone concentration was found in subjects with apo E3/4 and E4/4 genotype than in those with either apo E2/3 or E3/3. A similar trend was observed in the Chinese population, but this was not significant. However, in Chinese women the apo E3/4 and E4/4 genotypes were associated with lower %ucOC than were apo E2/3 and E3/3 genotypes (Yan *et al.*, 2004, 2005). The differences between countries in apo E genotype could explain some ethnic differences in osteoporosis risk, but these studies do not confirm the results by Saupe *et al.* (1993) (Kohlmeier *et al.*, 1998) that the apo E4 allele is associated with an increase in adjusted ucOC.

C. Vitamin K source and intake

Phylloquinone occurs in high amounts in green leafy vegetables and vegetable oils and margarines, while menaquinones are mainly found in liver, certain cheeses, and fermented soybean products. Besides the dietary sources of vitamin K, the human intestinal flora does also contribute to the vitamin K status. The contribution from the intestinal flora might, however, be much less than previously anticipated (Groenen-Van Dooren *et al.*, 1995).

The intake of vitamins K₁ and K₂ varies between different population groups. Intake of vitamin K₂ is high in some parts of Asia where the intake of fermented soy products is high, while phyloquinone is the primary source of vitamin K intake in most Western countries (Binkley and Suttie, 1995). Methods to measure vitamin K in its different forms in food have only recently been developed; therefore, many countries still do not have available information about the food content of vitamins K₁ and K₂. A few studies are available though, in which the intake of vitamin K has been investigated (Booth and Suttie, 1998; Booth *et al.*, 1996, 1999; Bolton-Smith *et al.*, 2000; Duggan *et al.*, 2004; Mensink and Beitz, 2004; Schurgers *et al.*, 1999; Thane *et al.*, 2006).

In the USA, the intake of phylloquinone has been estimated to range from 61 $\mu\text{g}/\text{day}$ to 210 $\mu\text{g}/\text{day}$ (Booth and Suttie, 1998), with a medium intake of 70–80 $\mu\text{g}/\text{day}$ (Booth *et al.*, 1999). In Britain (Thane *et al.*, 2006), Scotland (Bolton-Smith *et al.*, 2000), and Ireland (Duggan *et al.*, 2004), it has been estimated that the dietary intake is similar around 70 $\mu\text{g}/\text{day}$ (Thane *et al.*, 2006), while the intake of phylloquinones in the Netherlands and Germany is much higher, ~ 250 and 400 $\mu\text{g}/\text{day}$, respectively (Mensink and Beitz, 2004; Schurgers *et al.*, 1999). In the USA, UK, and Ireland, it was estimated that more than 50% of the populations investigated had phylloquinone intakes below the recommended 1 $\mu\text{g}/\text{kg}/\text{day}$ (Institute of Medicine, 2001; Thane *et al.*, 2006).

In general, it has been observed that intake increases with age (Bach *et al.*, 1996; Booth *et al.*, 1996, 1999; Thane *et al.*, 2006) probably because of a higher intake of green vegetables in the older age groups.

D. Measurement of vitamin K status

Various indicators have been used to assess vitamin K status in humans (Booth and Suttie, 1998). Of these, only one, prothrombin time, has been associated with adverse effects, while the others have been shown to respond to altered vitamin K intake, but the physiological significance of these alterations is unknown. The traditional method, determination of prothrombin time, however, is insensitive and cannot be used to measure changes in dietary vitamin K intake in healthy subjects. Other more sensitive methods have been introduced, for example, factor VII activity, phylloquinone concentration, PIVKA-II, ucOC, and urinary excretion of γ -carboxyglutamyl residues (urine-GLA). Factor VII activity has been used as a marker for vitamin K status because of its relatively short half-life (~ 6 h). However, the method is not useful if subjects are not receiving antibiotics, and therefore it cannot be used in healthy subjects (Institute of Medicine, 2001). Plasma or serum concentration of phylloquinone may also be used as a marker of vitamin K intake in populations, with phylloquinone as the primary dietary vitamin K source. Plasma and serum phylloquinone concentrations respond to changes in dietary vitamin K intake (Sokoll *et al.*, 1997). But because of the wide daily variation in vitamin K intake, a single plasma or serum phylloquinone concentration may not reflect habitual dietary intake (Institute of Medicine, 2001). Gla residues are excreted via urine after normal protein catabolism and may be used to assess vitamin K status. However, a number of studies have shown that it may take several days for the urinary Gla concentration to respond to alterations in dietary vitamin K intake (Ferland *et al.*, 1993; Suttie *et al.*, 1988). Furthermore, there seems to be an age-related response of urinary Gla to vitamin K intake.

PIVKA-II is formed when vitamin K intake is too low to maintain normal γ -carboxylation of prothrombin, and the dietary-induced changes

in PIVKA-II seem to be unaffected by age (Sokoll and Sadowski, 1996). A number of studies have shown abnormal PIVKA-II concentrations with low vitamin K intake ($<60 \mu\text{g/day}$), while higher vitamin K intakes ($>80 \mu\text{g/day}$) were associated with normal PIVKA-II concentrations (Booth *et al.*, 1999; Ferland *et al.*, 1993; Suttie *et al.*, 1988). However, the different studies used different assays to determine PIVKA-II and an adequate dose-response study for a single procedure is still lacking. Like PIVKA-II, ucOC may be a suitable indicator for vitamin K intake, and some would say even better (Sokoll and Sadowski, 1996). Its use will be discussed further in the following sections. But as with PIVKA-II, there are still too many technical problems with the assay, and the physiological significance of diet-induced changes is still not clarified (Institute of Medicine, 2001).

E. Undercarboxylated osteocalcin

The circulating concentration of ucOC has been reported to be a marker of hip fracture risk and a predictor of BMD (Booth *et al.*, 2000, 2003a; Jie *et al.*, 1996; Szulc *et al.*, 1993, 1996). The serum concentration of ucOC in un- or placebo treated subjects has been found to range from 7.5% to 50% in postmenopausal women with estimated intakes of phyloquinone ranging from $90 \mu\text{g/day}$ to $220 \mu\text{g/day}$ (Binkley *et al.*, 2000; Booth *et al.*, 1999, 2003b; Bügel *et al.*, 2007; Knapen *et al.*, 1989). Besides the variations in phyloquinone intake, the differences in %ucOC may also be because of the use of different assays in different studies. A few studies have investigated the dose response of ucOC to different intakes of phyloquinone (Binkley *et al.*, 2002; Bügel *et al.*, 2007). In one study, doses of 0, 200, and $500 \mu\text{g/day}$ were given as supplements in addition to the habitual diet for 6 weeks (Bügel *et al.*, 2007), and in the other study, doses of 250, 375, 500, and $1000 \mu\text{g/d}$ were given for 2 weeks (Binkley *et al.*, 2002). In both studies, phyloquinone supplementation decreased %ucOC dose dependently, but it seems that to reach maximal decrease in %ucOC, at least $1000 \mu\text{g}$ phyloquinone is needed per day (Binkley *et al.*, 2002; Bügel *et al.*, 2007).

IV. VITAMIN K RECOMMENDATIONS

It could be argued, and some researchers do argue, that the present dietary recommendations are too low to be sufficient for optimal bone health. The present adequate dietary intakes (AI) of vitamin K are 120 and $90 \mu\text{g/day}$ for men and women, respectively, and are based on the effects of vitamin K on the coagulation system (Institute of Medicine, 2001). Determination of the requirement of vitamin K is difficult, as it is almost

impossible to induce vitamin K deficiency on a vitamin K free diet. However, bacterial production of vitamin K is not sufficient to maintain normal vitamin K status. The Food and Nutrition Board questioned the alternative methods used to assess vitamin K status because of uncertainties as to their true physiological significance, as mentioned above. Therefore, the AI has been based on vitamin K intake in apparently healthy population groups using the prothrombin time essay. The conclusion, therefore, is that by restricting the vitamin K intake to levels which are almost impossible to achieve in a self-selected diet, it is not possible to impair normal hemostatic control in healthy subjects. As it is stated in the Nordic Nutrition recommendations, “it cannot be excluded, however, that this amount might be insufficient to fully support maximal osteocalcin γ -carboxylation in the elderly” (Nordic council of ministers, 2004). In fact, a number of studies have proven this statement correct (Booth *et al.*, 1999; Bügel *et al.*, 2007; McKeown *et al.*, 2002). In these studies, it was shown that the %ucOC was more than 20% at habitual phyloquinone intakes and that addition of phyloquinone could decrease the amount of %ucOC to half or less (Booth *et al.*, 1999; Bügel *et al.*, 2007).

Vermeer *et al.* (2004) has argued that the recommendations should be increased, as it seems that based on the bone markers at least 200–500 μg or more of dietary vitamin K is required per day for optimal carboxylation of OC.

V. ANTICOAGULATION TREATMENT AND RISK OF OSTEOPOROSIS

While some researchers are pleading for recommendations of higher intakes of vitamin K, others are using the conflicting results from studies with patients undergoing treatment with oral anticoagulants to argue against increasing the recommendations. While the use of oral anticoagulants during the first trimester of pregnancy clearly has deleterious effects on fetal bone development (Pettifor and Benson, 1975), it is unclear whether treatment with anticoagulants increases the risk of osteoporosis in adults. Some studies have shown an increased risk of osteoporosis with the use of oral anticoagulants (Caraballo *et al.*, 1999; Monreal *et al.*, 1991; Philip *et al.*, 1995; Sato *et al.*, 1997), while others fail to see any adverse effects on bone (Jamal *et al.*, 1998; Piro *et al.*, 1982; Rosen *et al.*, 1993). Low-dose warfarin has been shown to affect vitamin K status in both young (20–25 years) and older subjects (55–75 years). One milligram warfarin, together with 40 μg vitamin K₁ per day, increased the serum ucOC and PIVKA-II, while urinary GLA decreased within 11 days (Bach *et al.*, 1996). Addition of 154 μg vitamin K₁ parenterally to these subjects improved the PIVKA-II level.

So, anticoagulant treatment and vitamin K do interact and do have physiological consequence. But it is still unclear how and to what extent.

In a meta-analysis by [Caraballo *et al.* \(1999\)](#), it was concluded that long-term anticoagulant therapy was associated only with a modest increase in osteoporotic fracture risk. The use of oral anticoagulants was negatively associated with bone density in the ultradistal radius, but not in the spine or hip ([Caraballo *et al.*, 1999](#)). As Vermeer ([Vermeer and Hamulyak, 2004](#)) said: “It is remarkable that even after 50 years in which coumarin treatment has been widely used, the number of reported side-effects has remained remarkable low.” Vermeer suggests that the reason could be that there are back-up systems that are taking over when the functions of extrahepatic Gla proteins are eliminated by coumarin. Alternatively, that the proteins may be primarily involved in slow physiological processes so that their potential side effects become apparent only after decades rather than after months ([Vermeer and Hamulyak, 2004](#)).

VI. INTERACTIONS BETWEEN VITAMIN K AND VITAMIN D

An alternative explanation for the lack of a clear connection between the use of oral antiocoagulants and fracture could also be as has been suggested by some researchers, that the relationship with hip fracture reflects a combination of both poor vitamin K status and poor vitamin D status ([Booth *et al.*, 1999](#); [Szulc *et al.*, 1993](#)). In women with low BMD, the increase in %ucOC and BMD was larger when the vitamins were given in combination than when vitamin K was given alone ([Schaafsma *et al.*, 2000](#); [Ushiroyama *et al.*, 2002](#)). Animal and cell studies have shown that 1,25-dihydroxy vitamin D induces the synthesis of OC by promoting transcription of its gene ([Lian *et al.*, 1989](#)), while vitamin K is responsible for the posttranscriptional activation. *In vitro*, it has been shown that in the presence of vitamin D, menaquinones, enhances the vitamin D-induced Ca deposition and mineralization ([Koshihara *et al.*, 1996](#)). Furthermore, menaquinone was shown to induce OC mRNA expression and production in the presence of 1,25 dihydroxycholecalciferol, whereas alone they had no effect on mRNA expression ([Koshihara *et al.*, 1996](#)). In ovariectomized rats, vitamins D and K seem to act synergistically to reduce bone loss ([Matsunaga *et al.*, 1999](#)), while postmenopausal women with low vitamin K intake and high vitamin D intake were found to have an increased risk of hip fractures ([Feskanich *et al.*, 1999](#)). In theory, these findings can be explained from the action of the two vitamins on Ca homeostasis. Vitamin D acts as an inducer of bone resorption, and thus higher intake may result in an increased bone turnover and an increased urinary Ca excretion. Vitamin K,

however, may decrease the urinary Ca excretion as observed in both animals (Kobayashi *et al.*, 2002) and humans (Knapen *et al.*, 1993). Thus, despite high intakes of vitamin D, there may be an increased risk of hip fractures when vitamin K intake is low (Feskanich *et al.*, 1999). Furthermore, vitamin D induces synthesis of OC, but if vitamin K intake is low, the OC remains undercarboxylated, thereby increasing the levels of ucOC in blood and bone.

VII. DISCUSSION AND CONCLUSION

Overall, there are strong indications for an association between vitamin K status and optimal bone health. However, as long as the exact role of vitamin K is not known, a relationship between vitamin K intake and fracture risk needs to be established in order to increase the recommended levels.

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DIAGNOSIS OF OSTEOPOROSIS WITH VITAMIN K AS A NEW BIOCHEMICAL MARKER

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Abstract

Osteoporosis is a metabolic bone disease characterized by reduced bone quality and quantity. As a consequence, patients are at risk for fractures, subsequent immobility, and higher mortality especially among elder patients. Because of the high incidence of complications and the associated financial

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burden for the health system, new parameters for diagnostic and therapeutic purposes are urgently needed. In this regard, research focused on vitamin K as a biochemical bone marker has provided promising results. Vitamin K represents an important enzyme-cofactor for the posttranslational modification and activation of several proteins involved in bone metabolism. Vitamin K has been proven to be a valuable diagnostic as well as therapeutic parameter especially in osteoporosis. Patients with osteoporosis have been shown to have decreased levels of vitamin K. Further, regular intake of vitamin K may increase bone mineral density (BMD), thereby lowering the fracture risk. Yet vitamin K alone may not sufficiently indicate the mineral status of the bone. However, the usefulness of a combination of several biochemical bone markers as improved surrogate markers of bone metabolism has been shown recently. Therefore, this review will focus on the significance and importance of vitamin K for bone metabolism. Beyond this, aspects on the current and prospective use of vitamin K as well as other newly developed biochemical bone markers will be discussed. © 2008 Elsevier Inc.



I. INTRODUCTION

Osteoporosis is a multifactorial systemic metabolic disease with hormonal, genetic, and nutritional aspects. The National Institutes of Health (NIH) Consensus Panel defined osteoporosis as a skeletal disorder that is characterized by compromised bone strength and predisposes a person to an increased fracture risk ([National Institutes of Health, 2001](#)). The incidence of osteoporosis is extremely high particularly among the elderly and ranges around 50% among men and women above the age of 50 ([Cummings and Melton, 2002](#); [Kanis, 1994](#); [Lane, 2006](#); [Melton *et al.*, 1993](#)). This makes age a major predictor of the disease. The increase in life expectancy particularly in Western countries entails a considerable rise in costs for the treatment of osteoporosis and associated fractures. Osteoporosis and the numerous complications affiliated with it are becoming a major issue to the European public health system, causing expenses of ~€3.5 billion per year ([Götte and Dittmar, 2001](#); [Norris, 1992](#)). Beyond this socio-economic aspect, the individual psychosocial effects must not be forgotten. Osteoporotic fractures often involve a higher risk of mortality as well as functional deficits resulting in the need for long-term care and greatly constricted quality of life for the affected patients ([AWMF Online, 2006](#); [Cornell *et al.*, 2003](#); [Lorrain *et al.*, 2003](#); [Morris and Masud, 2001](#)). Therefore, efforts must be directed toward the further investigation of the disease pattern to define effective preventive measures and establish improved treatment strategies.

A. Mechanisms of bone loss in osteoporosis

Because of a decrease in bone mass and a deterioration of the overall quality of the bone, osteoporosis is associated with an increased fracture risk for the affected patients (Parkinson and Fazzalari, 2003). Beyond that, fractures are significantly exacerbated (Bonnaire *et al.*, 2005) and healing is often impaired in osteoporotic patients (Augat *et al.*, 2005). The deterioration of the bone's microstructure also results in decreased holding power of fixation systems, thereby greatly complicating the management of osteoporotic fractures (Cornell, 2003; Curtis *et al.*, 2005; Giannoudis and Schneider, 2006; Koithan and Oestern, 2005; Larsson and Bauer, 2002).

Postmenopausal osteoporosis and loss of bone mass during aging result from an imbalance between bone resorption and formation (Fig. 17.1A and B). The reasons for increased bone resorption lie in prolonged life spans of osteoclasts with delayed apoptosis and shortened working life spans of osteoblasts (Manolagas, 2000). At the same time, the reduced bone formation is an effect of a decrease in cell recruitment, precursor cell proliferation, and/or differentiation. Beyond that, a higher incidence of apoptosis occurring in osteoblasts has a detrimental effect on the structure of the bone (Fromigue *et al.*, 2004). Further reasons for low bone mass can be attributed to glucocorticoid therapy resulting in decreased bone formation. This leads to thinner, therefore weakened, trabecular walls and osteonecrosis. Rare genetic disorders such as inactivating mutations in the lipoprotein receptor-related protein 5 gene can lead to osteoporosis-pseudoglioma syndrome and idiopathic osteoporosis in men with low bone mass and impaired bone

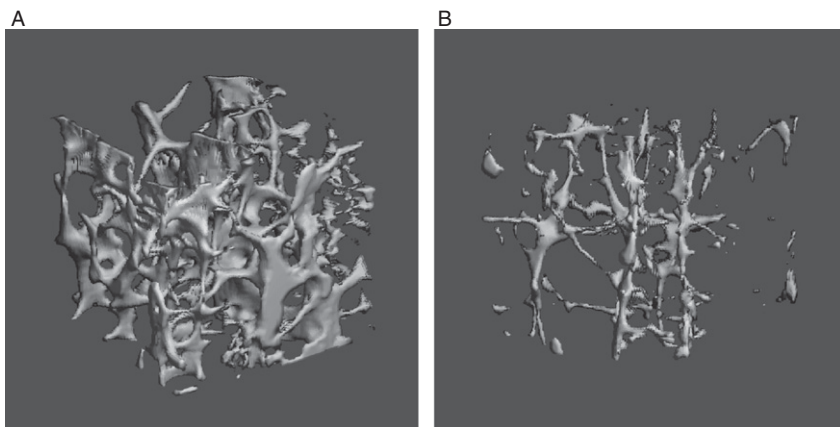


Figure 17.1 (A) 3D-Micro-CT reconstruction of a bone chip (1×1 cm) with μ CT 20 of the cancellous part of lumbar vertebra of a postmenopausal nonosteoporotic patient. (B) 3D-Micro-CT reconstruction of a bone chip (1×1 cm) with μ CT 20 of the cancellous part of lumbar vertebra of a postmenopausal osteoporotic patient.

formation (Ferrari *et al.*, 2005; Russell *et al.*, 2006). While the genes involved in the complex disorder of idiopathic osteoporosis are completely unknown, various physiological and pharmacological stimulators and inhibitors maintaining skeletal homeostasis and bone mass at steady-state level are known and have been reviewed recently (Harada and Rodan, 2003).

B. Growth factors, cell-based therapies, and transcriptional regulation increasing bone formation

Based on the different pathophysiological mechanisms underlying osteoporosis, treatment strategies for osteoporotic patients must not only decrease bone resorption, for instance, by applying antiresorptive bisphosphonates, but also increase bone formation and repair skeletal structures. Various growth factors are known to influence the recruitment and differentiation of osteoblasts. Among these, transforming growth factors (TGF), fibroblast growth factors (FGF), insulin-like growth factors (IGF), platelet-derived growth factors (PDGF), and bone morphogenetic proteins (BMP) can be found in bone matrix and have been extensively studied regarding the influence on bone cells and their precursors both *in vitro* and *in vivo* (Kanaan and Kanaan, 2006; Nakase and Yoshikawa, 2006; Regauer *et al.*, 2006; Simpson *et al.*, 2006; ten Dijke, 2006). However, because of the lack of specificity and far-ranging side effects concerning cell proliferation and survival on various other cell types, systemic administration of growth factors is currently not feasible. Nonetheless, autogenous bone marrow can be expanded *in vitro* for the treatment of osteoporotic patients using FGF-2 to facilitate this expansion or TGF- β and BMP to enhance the differentiation process prior to an application *in vivo* (Varkey *et al.*, 2006). Various cell-based therapies have been developed over the intervening years and will eventually find their way into widespread clinical application (Cancedda *et al.*, 2003; Caplan *et al.*, 1998; Wang *et al.*, 2006b). Beyond this, within the scope of local gene therapy, conditional overexpression of certain genes in selectively administered bone cells might allow to utilize the anabolic effects of some growth factors on bone *in vivo* or accelerate the healing of fractures without severe systemic side effects.

Transcriptional regulation of bone formation is a promising approach for future therapeutic strategies. A number of transcription factors that control osteogenesis and chondrogenesis have been identified (Akiyama *et al.*, 2002; Ducy *et al.*, 1997; Komori *et al.*, 1997; Otto *et al.*, 1997). Especially the role of runt-related transcription factor 2 (Runx2), formerly named Cbfa1, for osteoblastic differentiation was demonstrated in knockout animal models (Otto *et al.*, 1997; Vaes *et al.*, 2006; Xiao *et al.*, 2004) as well as in cleidocranial dysplasia in humans (Otto *et al.*, 2002; Vaughan *et al.*, 2002). The role of further transcription factors and cofactors on the osteogenic cascade such as distal-less homeobox 5 (Dlx5), msh homeobox homologue 2 (Msx2),

the Fos-family transcription factors, and the zinc finger-containing protein Osterix (Osx), which acts downstream of Runx2, has been reviewed by [Harada and Rodan \(2003\)](#). With a better understanding of the osteogenic cascade on the transcriptional level and new targets which are responsible for a high bone mass in humans such as low-density lipoprotein (LDL)-receptor-related protein 5 (LRP5), a coreceptor in the Wnt/Wingless pathway ([Baron et al., 2006](#)), new anabolic therapeutic concepts might be developed in future.

C. The role of nutrients in bone metabolism and the prevention of osteoporosis

[Yamaguchi \(2006\)](#) and [Palacios \(2006\)](#) have recently reviewed the influence of nutritional factors on bone health. Various proteins, minerals (calcium, phosphorus, magnesium, fluoride, zinc, copper, boron, manganese, potassium, iron), and vitamins D, C, A, B, especially vitamin K, have been shown to have an important influence on bone formation and might have preventive as well as therapeutic effects on osteoporosis.



II. THE IMPORTANT ROLE OF VITAMIN K IN BONE METABOLISM

This chapter will focus especially on vitamin K, delineating its role in bone metabolism and presenting it as a new biochemical marker to diagnose osteoporosis. The importance of vitamin K, a member of the 2-methyl-1,4-naphthochinons, as a therapeutic target in osteoporosis is well known ([Ryan-Harshman and Aldoori, 2004](#)) and has been recognized for the treatment of osteoporotic patients years ago. In studies administering vitamin K to osteoporotic postmenopausal women, the supplement was proven to result in an increase in bone mineral density (BMD; [Plantalech et al., 1991](#); [Vermeer et al., 2004](#)). Beyond this, other studies showed that the administration of vitamin K prevents bone loss and effectively reduces the incidence of fractures ([Shiraki et al., 2000](#)). However, these results are not consistent between research groups. In a study conducted by [Ishida and Kawai \(2004\)](#), no protective effect of vitamin K could be shown. Correspondingly, even though the effects of vitamin K on intracellular mechanisms of bone cells have been reviewed by [Iwamoto et al. \(2004\)](#), this field of research cannot be considered to be satisfactorily attended to in all respects. Various aspects need to be considered with regard to the effect of vitamin K on bone metabolism.

A. Vitamin K-dependent proteins in bone metabolism

Vitamin K acts as a cofactor of γ -carboxylase, a microsomal enzyme that carboxylates glutamic acid residues in proteins, especially osteocalcin (bone Gla protein) and matrix Gla protein (MGP; [Adams and Pepping, 2005](#); [Kinoshita *et al.*, 2007](#); [Shearer, 2000](#)). This posttranslational modification of vitamin K-dependent proteins is necessary for their functionality. In osteoporosis, it has been found that noncarboxylated osteocalcin is increased in vitamin K deficiency ([Szulc *et al.*, 1996](#)) and cannot bind to hydroxyapatite in bone ([Hauschka and Carr, 1982](#); [Price, 1985](#)). In contrast, supplementing vitamin K reduces undercarboxylated osteocalcin, bone resorption as well as urinary calcium secretion, and increases bone formation ([Braam *et al.*, 2003](#); [Feskanich *et al.*, 1999](#)).

Three isoforms of vitamin K are known: vitamin K₁ (phylloquinone), vitamin K₂ (menaquinones), and vitamin K₃ (menadione) ([Kaneki *et al.*, 2006](#)). Vitamins K₁ and K₂ are mainly found in vegetables or meat, milk, and cheese, respectively. Vitamin K₃ is a chemically synthesized derivate that is used for animal feed ([Stafford, 2005](#)). Whereas vitamins K₁ and K₂ have been considered to have similar effects on homeostasis, they assume different roles concerning bone metabolism. Especially menaquinone-7, an analogue of vitamin K₂, has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption ([Iwamoto *et al.*, 2006](#)). For vitamin K₁ no association between the intake of this supplement and either the BMD or the fracture risk, respectively, in perimenopausal and postmenopausal women could be demonstrated ([Rejnmark *et al.*, 2006](#)).

B. Does subclinical vitamin K deficiency increase the risk of fractures?

[Kaneki *et al.* \(2006\)](#) have recently reviewed the contribution of subclinical vitamin K deficiency to age-related bone loss and osteoporotic fractures. They discuss three main reasons to support their theory: (1) The proven association of increased levels of undercarboxylated osteocalcin, due to subclinical vitamin K deficiency, with an increased fracture incidence and low BMD ([Szulc *et al.*, 1994](#); [Vergnaud *et al.*, 1997](#)). (2) Different levels of vitamin K with low BMD and bone fractures in cross-sectional studies ([Booth *et al.*, 2004](#); [Hodges *et al.*, 1993](#)). (3) The correlation between vitamin K intake, fracture risk, and BMD in prospective cohort studies ([Booth *et al.*, 2003](#); [Feskanich *et al.*, 1999](#)). However, to date it remains unclear whether the increased incidence of fractures is attributable to BMD-dependent mechanisms ([Iwamoto *et al.*, 2006](#)) or to decreased bone quality being reflected in an impaired microarchitecture and a heightened fragility of bone ([Liu and Peacock, 1998](#)). Moreover, [Rejnmark *et al.* \(2006\)](#) could not show associations between the intake of vitamin K₁ and BMD of

the femoral neck or lumbar spine in a cross-sectional and longitudinal analysis in a Danish population. In addition, a logistic regression analysis did not reveal any association between the intake of vitamin K₁ and risk of fracture. In contrast, [Iwamoto et al. \(2004\)](#) discuss a positive effect of vitamin K₂ on BMD and a reduced risk of osteoporotic fractures. Altogether, the issue of vitamin K intake on preventing fractures remains controversial. Keeping in mind the varied effects of vitamin K₁ and K₂ on bone metabolism, further reasons for differing results of various studies can be attributed to differently chosen target populations and methods of measurements.

C. The calcification paradox

While vitamin K is involved in the promotion of bone mineralization, vitamin K-dependent proteins play an important role in the prevention of arterial calcification in atherosclerosis ([Hofbauer et al., 2006](#); [Spronk, 2006](#)). Arterial calcification shares many similarities with bone metabolism and is discussed to be an active, cell-controlled process. Two forms are known, intimal calcification in the context of atherosclerosis and medial calcification (Mönckeberg's arteriosclerosis) which occurs independently of atherosclerosis. To date there is still a debate whether intimal calcification has a stabilizing effect on atherosclerotic plaques or promotes ruptures of plaques. Apparently early or intermediate stages of calcification increase plaque vulnerability to rupture, whereas extensive calcification of atherosclerotic plaques might prevent rupture ([Spronk, 2006](#)).

MGP and osteocalcin (Gla Protein) are known to be regulators of tissue mineralization. While osteocalcin is linked to bone metabolism and osteoporosis, MGP plays a major role in the inhibition of soft tissue calcification. [Luo et al. \(1997\)](#) showed impaired bone growth as well as extensive medial calcification of the aorta in MGP-deficient mice. Undercarboxylation of MGP has been shown to be associated with intimal as well as medial vascular calcification ([Schurgers et al., 2005](#)). While vitamin K epoxide reductase complex subunit 1 (VKORC1) is the key protein in the vitamin K cycle (reviewed in [Oldenburg et al., 2006](#)), [Wang et al. \(2006a\)](#) present a polymorphism in this subunit to be associated with a higher risk of arterial vascular disease. Other potential calcification inhibitors besides MGP are fetuin-A and pyrophosphates. For the genes of MGP, osteopontin, fetuin-A, Smad6, Klotho, and osteoprotegerin (reviewed in [Hofbauer et al., 2006](#)), a correlation between skeletal and vascular phenotypes in knockout mice has been shown.

D. Vitamin K as diagnostic parameter in osteoporosis

Based on the various important functions of vitamin K in bone metabolism, its potential significance as a diagnostic tool has emerged among clinical and basic researchers in recent years. The importance of vitamin K as a

therapeutic target in osteoporosis is well known (Ryan-Harshman and Aladoori, 2004). Previous studies investigated the usefulness, efficacy, and power of vitamin K for assessing the bone metabolism and possibility for the diagnosis of osteoporosis (Hodges *et al.*, 1993; Kanai *et al.*, 1997; Vermeer *et al.*, 1995, 1997). Despite these studies, its suitability as a diagnostic parameter has not been well established. Therefore, to increase the suitability and sensitivity of vitamin K, the combination with several other biochemical bone markers (see below) and their multidimensional analysis by adequate mathematical processes (logistic regression, neuronal networks, fuzzy methods) may be useful (Keller *et al.*, 1998; Rommelfanger, 1994).

We have recently published a study where we aimed to evaluate the usefulness and potential of a multidimensional analysis of vitamin K as a biochemical bone marker for osteoporosis (Heiss *et al.*, 2004). Using data records of women between 28 and 74 years of age, we analyzed the usefulness of vitamin K in combination with other parameters to differentiate between premenopausal women and postmenopausal nonosteoporotic and osteoporotic women. BMD was determined by quantitative computed tomography (qCT) of the lumbar spine using the *T*-score, and levels of vitamin K and other biochemical markers of bone formation and resorption (AP, bAP, OC, ucOC, PICP, PYD, DPD, NTx, BSP) were analyzed. Vitamin K level was found to be significantly lower in osteoporotic patients when compared to that in nonosteoporotic as well as premenopausal women (Fig. 17.2). Although statistically not significant, AP, bAP, and NTx were higher in the osteoporotic group, while OC, ucOC, PICP, and BSP were lower in osteoporotic women (Table 17.1). Further, the odds-ratio indicating the possibility of a correct diagnosis if this test is applied revealed 16.7 for vitamin K, which was significantly higher than that for other bone indices. With a sensitivity of 64% and a specificity of 82%, vitamin K reached the highest AUC-score in the ROC analysis (Table 17.2, Fig. 17.3). Surprisingly, by combining vitamin K and AP, bAP and PYD, AUC-scores were even higher (>0.9), while the parameter combination of vitamin K/PYD and vitamin K/bAP demonstrated a sensitivity of 75–88% with a specificity of more than 82%. These data suggest that a combination of vitamin K with other biochemical bone indices may be a useful tool for assessing bone metabolism, especially in metabolic bone diseases like osteoporosis. However, especially in view of the discrepancy between various study results, it is more than evident that further investigations are needed to actually evaluate the suitability of vitamin K for the prophylaxis and diagnosis as well as its possible benefits for the treatment of metabolic bone diseases such as osteoporosis.

E. Vitamin K as therapeutic target in osteoporosis

The rationale behind a treatment with vitamin K in osteoporosis is based on many clinical as well as experimental and preclinical animal studies. While Rejnmark *et al.* (2006) did not show an effect of vitamin K1 intake on

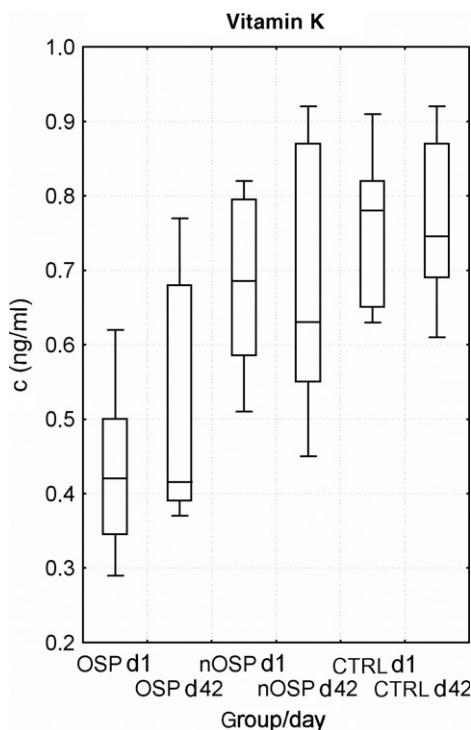


Figure 17.2 Box plot graph of vitamin K concentration in groups nOSP (postmenopausal nonosteoporotic), OSP (postmenopausal osteoporotic), and CTRL (premenopausal control) at two different time points (day 1 and 42). Bottom to top: 5/25/50/75/95% percentile. Published in *Biomarkers* 2004; 9(6), 479–488.

fracture risk and BMD in perimenopausal women, Ryan-Harshman and Aldoori conclude from 10 different clinical trials that the dietary intake of vitamin $K_1 < 100 \mu\text{g}$ daily is not optimal for bone health. In addition, the D-BAVIS study (Dundee Bone and Vitamins Intervention Study) showed that combined dietary supplementation with vitamin K_1 and vitamin D_3 significantly improved BMD and may be beneficial for groups with a high risk of osteoporosis-related complications (Bolton-Smith *et al.*, 2001).

Phylloquinone (vitamin K_1) constitutes a major part of vitamin K intake in Western countries, whereas in other countries, particularly in Japan, “natto,” a fermented soybean food, contains a large amount of menaquinone-7 (vitamin K_2) and has a large effect on the status of circulating vitamin K (Kaneki *et al.*, 2001). The positive effect of vitamin K_2 on bone metabolism has been extensively reviewed by Iwamoto *et al.* (2003a) in experimental and clinical settings. Moreover, Yamaguchi (2006) reviews the influence in both settings and also concludes that vitamin K_2 has been

Table 17.1 Concentration (mean and 5%/95% percentile of concentration) of biochemical bone markers at day 1 and 42 in postmenopausal nonosteoporotic (nOSP) and postmenopausal osteoporotic (OSP) patients

Biochemical bone markers	Mean concentration at days 1 and 42 (5%/95% percentile)		
	nOSP	OSP	Difference
bAP	18.3 14.6–26.6	20.5 12–22.7	n.s.
ucOC	4.9 2.5–6.3	4.0 1.9–6.6	n.s.
PICP	81.2 63–142	74.2 61–107	n.s.
PYD	99 83–116	110 99–137	<i>p</i> = 0.04
DPD	25.1 19.3–34.1	29.8 28.0–41.1	<i>p</i> = 0.01
NTx	51.3 36–93	53.3 38–98	n.s.
BSP	8.0 5.2–10.5	6.2 5.0–16.4	n.s.
Vitamin K	0.633 0.52–0.86	0.408 0.36–0.65	<i>p</i> = 0.01

bAP, bone alkaline phosphatase; ucOC, undercarboxylated osteocalcin; PICP, procollagen type I carboxyterminal propeptide; PYD, pyridinoline; DPD, deoxypyridinoline; NTx, N-terminal cross-linked telopeptide of type I collagen; BSP, bone sialo protein.

Mann–Whitney’s U-test was used to compare postmenopausal nonosteoporotic (nOSP) versus postmenopausal osteoporotic (OSP) women.

demonstrated to stimulate osteoblastic bone formation while inhibiting osteoclastic bone resorption at the same time.

The recommended dietary vitamin K intake has been phrased as a result of a closed workshop of vitamin K experts: It was concluded that a dietary intake of vitamin K between 200 and 500 $\mu\text{g}/\text{day}$ might be beneficial for the overall bone health. The same benefits might be achieved by supplements of ~ 100 $\mu\text{g}/\text{day}$ with a higher bioavailability of supplements. The supplementation of vitamin K should be combined with vitamin D and minerals. These additive effects of vitamins K and D have been addressed in many studies and are also reviewed in [Iwamoto *et al.* \(2003b\)](#).

The safety range of vitamin K is wide with no reports of adverse effects or hazards due to ingestion of natural vitamin K ([Weber, 2001](#)). A potential problem might arise from combined treatment with oral anticoagulants. However, doses of vitamin K supplements below 100 $\mu\text{g}/\text{day}$ have been shown not to influence the stability of anticoagulation in patients on oral

Table 17.2 Biochemical bone parameter combinations with vitamin K and their sensitivities/specificities of parameter combination

Combination of biochemical bone parameters	n	Sensitivity	95% Confidence interval	Specificity	95% Confidence interval
bAP/ Vitamin K	21	Day 1 7/8	53%	Day 1 10/11	64%
		88%		91%	
		Day 42 6/8	40%	Day 42 9/10	53%
		75%		90%	
PYD/ Vitamin K	21	Day 1 7/8	53%	Day 1 9/11	53%
		88%		82%	
		Day 42 6/8	40%	Day 42 9/11	53%
		75%		82%	

PYD, pyridinoline; bAP, bone alkaline phosphatase.

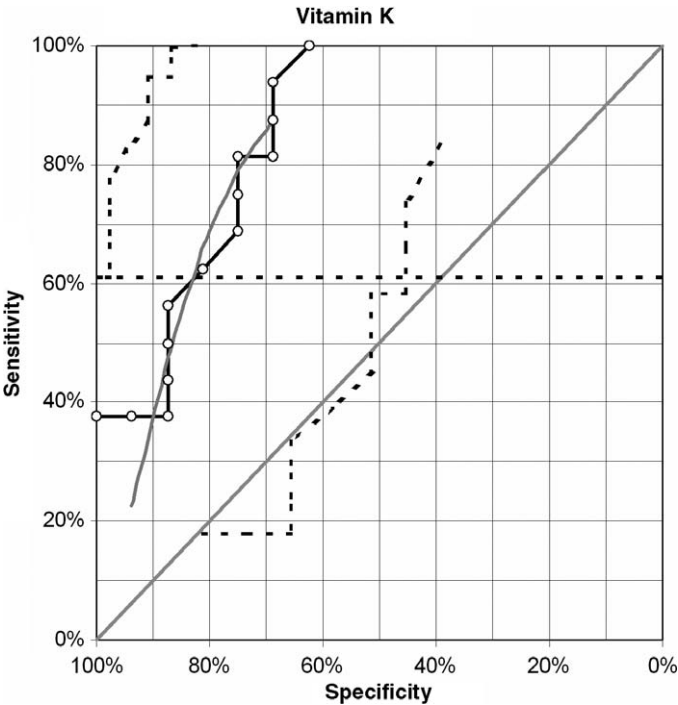


Figure 17.3 ROC analysis for vitamin K as biochemical marker in osteoporosis (black solid line). Gray line in ROC curve: trend line. Space within dashed lines: 90% confidence interval. Gray diagonal: ROC curve of lacking discrimination. Published in *Biomarkers* 2004; 9(6), 479–488.

treatment. In healthy individuals even a high consumption of vitamin K₁ or K₂ appears to have minimal risks and high doses have been used without observing adverse effects.

III. BIOCHEMICAL MARKERS AS INDICES OF BONE TURNOVER

As mentioned earlier, biochemical bone markers are metabolites of bone turnover that can be used to characterize the metabolic status of bone by measuring their concentration in serum and urine (Bikle, 1997; Christenson, 1997; Delmas, 1996; Dominguez *et al.*, 1998). Since vitamin K is closely related to biochemical bone markers, their clinical significance and relevance will be discussed here shortly. During the last 10–20 years, these bone indices were mainly used for monitoring therapeutic responses in bone diseases. However, their usefulness for the clinical diagnosis of bone diseases, especially osteoporosis, remains to be elucidated. Facilitated by the findings of new biochemical bone indices [e.g., aminoterminal telopeptide (NTx), bone sialo protein (BSP), and vitamin K], it has been shown that osteoporosis may result in increased levels of NTx and cross-links DPD/PYD as reviewed in Bollen *et al.* (1997), Delmas *et al.* (2000), and Looker *et al.* (2000). However, because of controversial reports in the literature, their suitability remains highly controversial (Hodges *et al.*, 1993; Vermeer *et al.*, 1995, 1997). Hence, there is still an urgent need for a suitable biochemical marker profile for prophylaxis and diagnosis of bone diseases, especially osteoporosis.

Just recently a new potential application of biochemical bone markers has emerged, that is, the usefulness of bone markers to assess fracture healing. When a fracture occurs, increased bone resorption and formation are needed to facilitate bone repair and fracture healing. Radiographs are the only method currently used to visualize and evaluate fracture repair. Because bone turnover is increased during fracture healing, biochemical markers of bone metabolism may be useful as surrogate parameters for monitoring fracture healing. In previous studies, it has been shown that markers of bone resorption in patients with vertebral or hip fractures are increased at an early stage of fracture healing (Bollen *et al.*, 1997; Hoshino *et al.*, 1998) while biochemical markers of bone formation increased at a later stage (Emami *et al.*, 1999; Kurdy *et al.*, 1998; Ohishi *et al.*, 1998; Sharland and Overstall, 1978). However, whether and how BMD, gender, fracture localization, or method of treatment affects these markers during fracture healing is largely unknown. Therefore, we have recently investigated the effects of a reduced BMD on fracture healing as indicated by levels of biochemical bone markers, especially in postmenopausal patients

(Hoesel *et al.*, 2005). In this study, biochemical markers of bone resorption increased in a time-dependent pattern, reflecting fracture healing postoperatively. Low BMD and female gender resulted in higher levels of bone markers. Further, parameters like fracture type and localization as well as way of operative treatment clearly had an impact on fracture repair as indicated by different elevations of bone indices (especially NTx). Most to our surprise, however, osteoporotic patients showed higher levels of NTx when compared to patients with normal BMD. This may indicate that the healing capability of bone is not impaired even by a low BMD. Taken together, the measurement of biochemical bone markers seems a promising tool to closely monitor fracture healing. Bone markers seem to reflect the ongoing fracture repair process and may provide important information about healing progress.

In conclusion, there is an increasing interest in biochemical parameters of bone metabolism for the diagnosis of osteoporosis. They may be, in contrast to osteodensitometric imaging, a valuable additional tool for the short-term evaluation of the “bone status.” Those markers and especially the here presented “new marker” combination of vitamin K may be helpful to screen and diagnose osteoporosis as well as to evaluate the associated bone loss and future fracture risk. Additionally, vitamin K may be useful to monitor therapeutic responses in osteoporotic patients.

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HEPATOCELLULAR CARCINOMA AND VITAMIN K

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Abstract

On the basis of reports of the antitumor effects of vitamin K on various cancers, we clinically investigated the suppressive effects of vitamin K2 on tumor recurrence after curative treatment for hepatocellular carcinoma (HCC). Our results showed that vitamin K2 administration significantly suppressed HCC recurrence. Our laboratory findings revealed that the inhibitory effect of vitamin K2 against HCC cell growth was generated by suppressing cyclin D1 expression through inhibition of NF- κ B activation. © 2008 Elsevier Inc.

I. INTRODUCTION

Vitamin K (VK) is a fat-soluble vitamin involved in blood coagulation and bone metabolism. In recent years, its antitumor effects have been closely examined. VK has been shown to suppress cancer growth and induce apoptosis and differentiation in various cancer cells, including leukemia cells and hepatocellular carcinoma (HCC) cells. VK2 is clinically used to suppress the onset of leukemia from myelodysplastic syndrome. Long-term

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administration of VK2 as a therapy for osteoporosis has established its clinical safety.

Here, we review previous studies of the anticancer effects of VK and introduce our recent laboratory data regarding the inhibitory effect of VK2 on HCC cell growth. Finally, we report a clinical study on the preventative effects of VK2 on posttreatment tumor recurrence.

II. ANTITUMOR EFFECTS OF VK

There are three forms of VK: VK1 (phytonadione), which is produced by plants and exists naturally; VK2 (menaquinone), which is produced by certain bacteria; and VK3 (menadione), which is chemically synthesized.

Research on the antitumor activities of VK began in the 1980s following a study documenting that VK3 suppressed cell growth and induced apoptosis (Chlebowski *et al.*, 1985). Since then, various studies have suggested that the antitumor activities of VK3 may be mediated by (1) the redox cycle, (2) cell cycle-related proteins, and (3) oncogenes. However, because of safety concerns, VK3 is not used clinically at this point in time. Although VK1 possesses hardly any antitumor activities, studies in recent years have confirmed that VK2 suppresses tumor cell growth, similar to VK3. Sakai *et al.* (1994) found that VK2 was able to induce leukemia cell differentiation and apoptosis. After finding that the same effects could be achieved with geranylgeranylacetone, which has the same structure as the side chain of VK2, the authors concluded that the side chain plays an important role. Furthermore, using leukemia cells obtained from a myelodysplastic syndrome patient, Yaguchi *et al.* (1998) showed that VK2 induced apoptosis via caspase-3. Subsequent clinical studies have shown that VK2 is effective against myelodysplastic syndrome.

In HCC patients, abnormal prothrombins [des- γ -carboxy prothrombin or protein induced by VK absence or antagonist-II (PIVKA-II)] are detected at high frequencies in the blood plasma. Hence, many studies have documented the suppressive effects of VK on the growth of liver cancer cell lines. Similar to other cancer cell types, the strongest suppressive effects are found for VK3, followed by VK2 and VK1, in that order (Hitomi *et al.*, 2005). Studies have shown that VK3 and VK2 have different mechanisms of growth suppression. Wang *et al.* (1995) found that addition of a catalase impeded the suppressive effects of VK3 on cancer growth, and therefore concluded that VK3 induced cell damage by producing free radicals (oxidative stress), since the suppressive effects of VK2 were not hindered by catalase or mediated by oxidative stress. Following experiments using new liver cancer cell lines, Bouzahzah *et al.* (1995) reported the probability that VK2 induced the expressions of *c-jun* and *c-myc*, thereby

elevating apoptosis, prothrombin, and other VK-dependent proteins and suppressing cancer growth.

III. MECHANISM OF GROWTH INHIBITION OF HCC CELLS BY VK2

VK2 has been shown to induce cell cycle arrest at G1/S, leading to growth inhibition of hepatoma cells both *in vivo* and *in vitro* (Hitomi *et al.*, 2005; Liu *et al.*, 2007; Otsuka *et al.*, 2004; Ozaki *et al.*, 2007; Wang *et al.*, 1995; Yoshiji *et al.*, 2005). The expression levels of several growth-related genes, such as cyclin D1 and cyclin-dependent kinase 4 (Cdk4), and cyclin-dependent kinase inhibitors, including p21 and/or p27, are altered in the growth-inhibitory process induced by VK2 (Liu *et al.*, 2007; Ozaki *et al.*, 2007; Wang *et al.*, 1995; Yoshiji *et al.*, 2005). One report (Matsumoto *et al.*, 2006) demonstrated that VK2 induced Hep3B cells to undergo apoptosis through activation of AP-1, while other studies did not detect apoptosis after treatment with VK2 alone.

Although the growth inhibition of HCC cells by VK2 is well documented, relatively high concentrations of VK2 (around the micromolar order) are required to inhibit HCC cell growth *in vitro*. Several studies have tested combinations of VK2 with other reagents used as anticancer reagents, such as interferon (IFN), 5-fluorouracil (5-FU), or retinoids (Kanamori *et al.*, 2007; Nakamura *et al.*, 2006), to enhance the antitumor effects of VK2. Nakamura *et al.* (2006) treated HCC cells with VK2 in combination with IFN- α or 5-FU, but found that VK2 could not increase the growth inhibitory effects of 5-FU and/or IFN. Retinoids, analogs of vitamin A, are known to possess antitumor effects. Kanamori *et al.* (2007) treated Huh7 cells with VK2 combined with acyclic retinoid (ACR) and found that VK2 plus ACR synergistically inhibited the growth of Huh7 cells.

These reports have also elucidated the signaling pathways involved in the actions of VK2. In the case of apoptosis induced by a combination of VK2 and ACR (Kanamori *et al.*, 2007), VK2 was found to inhibit Ras activation and phosphorylation of extracellular signal-regulated kinases (ERK) as well as phosphorylation of RXR. The presence of VK2 also enhanced RARE- and RXRE-containing promoter activities induced by ACR, although VK2 alone did not have any effects on these promoter activities. Analysis of cyclin D1 regulation by VK2 revealed that VK2 inhibited cyclin D1 promoter activity in a tumor necrosis factor- α (NF- κ B)-dependent manner (Ozaki *et al.*, 2007). Further analysis of the upstream signals for NF- κ B activation identified the inhibition of I κ B kinase activity by VK2. VK2 also inhibited the NF- κ B activation induced by inflammatory cytokines, such as TNF- α and interleukin-1 (IL-1), and the tumor-promoter 12-tetradecanoylphorbol-13-acetate (TPA). Aberrant expression of NF- κ B has been reported in various malignant

neoplasms, including HCC, and is linked to the onset and progression of tumorigenesis. Therefore, it is now regarded as a potential therapeutic target (Karin *et al.*, 2002).

VK2 is also known to be involved in bone metabolism, since it inhibits osteoclastic activity, and has been used for the treatment of osteoporosis in Japan (Orimo *et al.*, 1998). Takeuchi *et al.* (2000) reported that VK2 suppressed the expression of receptor activator of NF- κ B (RANK) mRNA and osteoclastic activity in murine bone marrow cells. These findings support the inhibitory effects of VK2 on NF- κ B activation, since the RANK/RANKL system also mediates NF- κ B activation. Cao *et al.* (2001) reported that cyclin D1 is one of the downstream targets of RANK/RANKL signaling, and in turn cyclin D1 was recently shown to regulate RANK expression (Fu *et al.*, 2005). Since both NF- κ B and cyclin D1 are known to regulate cellular migration (Kim *et al.*, 2006; Li *et al.*, 2006), the inhibition of both NF- κ B and cyclin D1 by VK2 may contribute to the suppression of HCC cell invasion.

While the ligands for VK have been explored, the transcription factor steroid and xenobiotic receptor (SXR) was found to bind VK2 and mediate gene expression in an osteosarcoma cell line (Tabb *et al.*, 2003). Interestingly, this research group further demonstrated that SXR is abundantly expressed in the liver and reciprocally regulates NF- κ B-regulated gene expression (Zhou *et al.*, 2006). These findings are intriguing, since they suggest a possible role for VK2 in the regulation of hepatic NF- κ B activation, which plays critical roles in inflammatory responses. A separate study by Ohsaki *et al.* (2006) also linked VKs to inflammation by showing that VK deficiency enhanced the expression levels of genes involved in acute inflammatory responses in the rat liver, while dietary supplementation with VK suppressed LPS-induced inflammatory responses.

Otsuka *et al.* (2004) reported that VK2 inhibited the growth of HCC cells as well as their invasiveness via protein kinase A (PKA) activation and subsequent inhibition of Rho activation. They also demonstrated activation of the transcription factors AP-2, USF-1, and CREB in HCC cells by showing nuclear accumulation of Ser-phosphorylated CREB, although the roles of these factors in VK2-induced suppression of cell growth and invasion are not known.

Besides VK2, other VK analogs are known to inhibit tumor cell growth and induce apoptosis. Carr *et al.* (2002) reported that a synthetic VK analog, designated compound 5, inhibited Cdc25A phosphatase activity and reduced HCC cell growth. However, the mechanisms of compound 5 seem to differ from those of VK2, since VK2 does not affect Cdc25A phosphatase activity or induce apoptosis in HCC cells.

Collectively, VKs have been shown to have diverse effects on the phosphorylation states of various proteins (Saxena *et al.*, 1997), although the mechanisms by which VK2 modulate protein kinases and/or

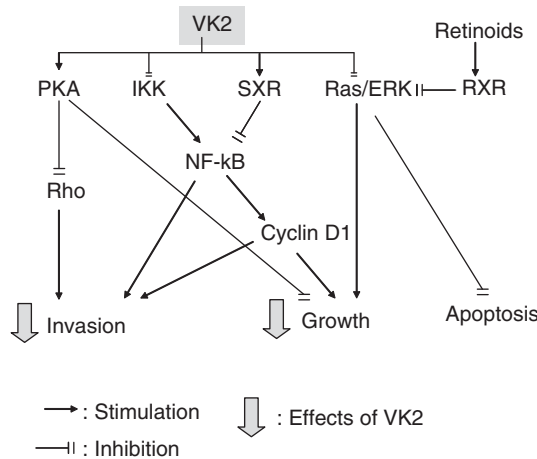


Figure 18.1 Inhibitory effects of vitamin K2 (VK2) on hepatocellular carcinoma (HCC) cell growth or invasion.

phosphatases remain to be elucidated. Therefore, VK2 may reduce the growth and invasion of HCC cells through the modulation of protein kinases/phosphatases (Fig. 18.1).

IV. ANALYSIS OF TUMOR RECURRENCE SUPPRESSION FOLLOWING LIVER CANCER TREATMENT

Despite recent progress in diagnosis and therapy, HCC remains among the cancers with the poorest prognoses. The primary reason for this is field cancerization, that is, multicentric carcinogenesis of HCC. In other words, since cancer can arise in all parts of the liver in chronic hepatitis, and liver cirrhosis in particular, even after treatment of the initial tumor, tumor recurrence in other areas occurs in 20–25% of HCC cases.

To ascertain the suppressive effects of VK2 on posttreatment tumor recurrence, we carried out a clinical study involving 61 patients with HCC who had undergone various treatments and been diagnosed as complete remission by dynamic computed tomography (CT). These patients were randomly divided into two groups: 32 patients in the VK2 group and 29 patients in the control group. The patients in the VK2 group were treated with 45 mg/day of menatetrenone (a VK2 analog; Eisai Co., Ltd., Tokyo, Japan). The HCC recurrence and survival rates were then compared between the two groups. The 1-, 2-, and 3-year recurrence rates were 55.2%, 83.2%, and 91.6%, respectively, for the control group and 12.5%,

39.0%, and 64.3%, respectively, for the VK2 group. Hence, VK2 administration appeared to significantly suppress tumor recurrence. A Cox proportional hazard model analysis showed that the HCC recurrence risk for the VK2 group declined to one-third of the level in the control group (0.322, $p = 0.0005$). The 3-year survival rates for the VK2 and control groups were 87.0% and 64.0%, respectively, with the VK2 group showing significantly better survival ($p = 0.05$). No adverse reactions, such as liver dysfunction, were seen in the VK2 group, and none of the patients withdrew because of adverse reactions during the observation period.

These findings suggest that VK2 administration is a safe and effective treatment for suppressing HCC recurrence and improving the prognosis (Mizuta *et al.*, 2006).

V. FUTURE TASKS

Although various studies of the anti-HCC effects of VK have been performed, many fundamental issues remain unclear, including its migration to and accumulation in the tumor tissue, its uptake mechanisms (e.g., receptors), and its subsequent signal transduction.

Clinically, the currently available VK preparations (VK1 and VK2) do not appear to exhibit marked antitumor effects when administered alone.

For these reasons, future clinical research on the anti-HCC effects of VK should undertake the following tasks: (1) examine the preventive effect of VK against HCC occurrence in high-risk patients using a multicenter double-blind study involving numerous subjects, (2) investigate coadministration of VK with other proven anticancer agents, and (3) develop a new VK preparation with a modified side chain.

It is our belief that continuing studies on VK will provide more detailed information on the issues raised above.

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