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ROLE OF VITAMIN K IN BONE METABOLISM

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ABSTRACT

Vitamin K is a cofactor required for the formation of γ-carboxyglutamate (Gla) residues in proteins. Osteoblasts produce at least three different Gla-containing proteins: osteocalcin, matrix Gla-protein, and protein S. After cellular secretion of these proteins, the main part of each remains bound to the hydroxyapatite matrix in bone, but their function remains unclear. Part of the newly synthesized osteocalcin is also set free into the bloodstream, where it may be used as a diagnostic marker for bone formation. Several studies have demonstrated that a poor vitamin K status is associated with an increased risk of osteoporotic bone fractures. Whether vitamin K supplementation will reduce the rate of bone loss in postmenopausal women remains a matter of debate.

INTRODUCTION

Several compounds found in nature have vitamin K activity, i.e. the ability to correct the bleeding tendency induced by nutritional vitamin K deficiency. The two most abundant forms of vitamin K are phylloquinone (vitamin K_1) and menaquinone (vitamin K_2). If not otherwise specified, the term vitamin K is used to describe a compound with vitamin K activity. The characterization of the previously unknown amino acid γ-carboxyglutamic acid (Gla) has led to the elucidation of the function of vitamin K in mammals (58, 91): to serve as a cofactor during the posttranslational carboxylation of glutamate into Gla. Most of the early observations were made with the blood coagulation factor prothrombin, and the importance of Gla residues for the enzymatic activity of this protein was demonstrated by comparing normal prothrombin with that circulating in animals treated with vitamin K antagonists (coumarin derivatives). In the latter case, the apparent prothrombin was functionally inactive because it lacked the 10 Gla residues present in normal prothrombin (92). Proteins missing one or more Gla residues are called undercarboxylated, or descarboxy, proteins.

SOURCES OF VITAMIN K FOR HUMANS

Phylloquinone

Phylloquinone is the only K vitamer synthesized by green plants and algae and is a major dietary form of vitamin K. In a recent review, Booth et al compared the available data in the literature (7). Although the amount of phylloquinone in food varies with soil and growth conditions, geographical differences, and time of harvesting, the following approximate ranges may be given: green leafy vegetables, 1000–8000 μg/kg; other vegetables and fruit,

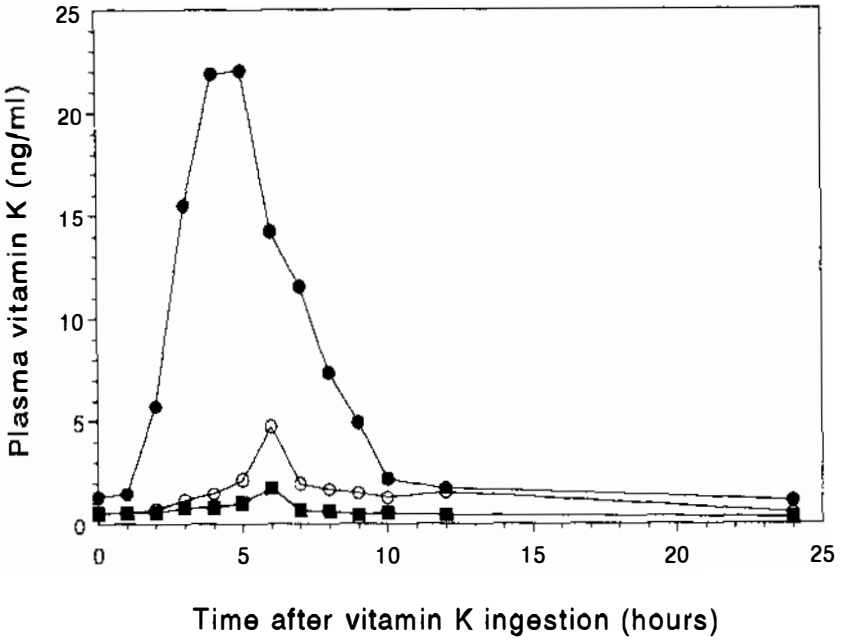

Figure 1 Absorption of vitamin K in a human volunteer. After an overnight fast, 1 mg phylloquinone was given either as a pharmaceutical preparation (Konakion®, Hoffmann–La Roche, Basel, Switzerland) (*solid circle*), in 250 g of spinach + 25 g of butter (*open circle*), or in 250 g of spinach without additives (*solid square*). Plasma vitamin K was determined according to Hart et al (30).

10–500 μg/kg; dairy produce, 3–70 μg/kg (strongly dependent on fat content); and grains, 0.5–70 μg/kg. The bioavailability of phylloquinone from these foods is unknown but must be expected to vary considerably; in green vegetables the vitamer is tightly bound to the thylakoid membranes of the chloroplasts (51). The gastrointestinal extraction from green vegetables is probably less efficient than that from foods like dairy produce in which the phylloquinone is solubilized in the fat component, where it may be absorbed without membrane degradation. Furthermore, the efficacy of vitamin K absorption from the intestinal lumen depends on the stimulation of secretion of bile salts and pancreas lipase by ingested fats. The effect of the source of vitamin K and of the concomitant ingestion of fat on its absorption is depicted in Figure 1, where we compare the appearance of phylloquinone in the circulation following an overnight fast with the subsequent consumption of 1 mg phylloquinone in the form of a solubilized pharmaceutical preparation, in 227 g of cooked spinach + 25 g butter, or in 227 g of cooked spinach without fat. In this

example, absorption from the pharmaceutical preparation (as determined by the area under the peak) was 8 times better than that from spinach + butter and 33 times better than that from spinach alone. The vitamin K content of various foods is therefore insufficient to determine whether a subject's vitamin K intake is adequate.

Menaquinones

The term menaquinone refers to a series of vitamin K compounds with poly-unsaturated aliphatic side chains of varying length. These compounds are generally referred to as MK-n, where n is the number of isoprenoid residues of which the side chain is composed. Natural menaquinones ranging from MK-4 to MK-13 are produced by bacteria, where they are tightly bound to the cytoplasmic membranes. In vitro, phylloquinone and various menaquinones are comparably active as cofactors for the hepatic vitamin K–dependent γ-glutamylcarboxylase (10), and like phylloquinone, menaquinones have vitamin K activity in vivo after oral ingestion (26). High menaquinone concentrations (up to 300 μg/kg) were found in yogurt, cheese, and butter (35, 89), as well as in natto (>9000 μg/kg), a dish prepared from fermented soy beans that is very popular in Japan (84). Moderate amounts of menaquinone were found in animal tissues such as fish, meat, and eggs (35). Although meats like beef and chicken mainly contain MK-4 (34 and 90 ng/g, respectively), higher menaquinones (MK-8 and MK-9) are the predominant forms of vitamin K in the liver (89). The intestinal flora also produces substantial amounts of menaquinones, but the extent to which these help satisfy mammalian vitamin K requirements remains under debate (11, 12, 52, 92b).

Bioavailability of Vitamin K

The lipophilicity of phylloquinone and of the most abundant menaquinones (MK-7 to MK-11) varies considerably (37, 38, 88, 96), potentially resulting in different tissue distribution and/or in tissue-specific activity of the various vitamers. In human bone, substantial amounts of menaquinones have been found, but how they are stored and whether they are available for osteoblast carboxylase remain unknown (38). The role of menaquinone stores in liver is also unclear. Several authors have confirmed that the majority of rat and human hepatic vitamin K stores consist of long-chain menaquinones (88, 96, 98). In rats with severe hypoprothrombinemia due to nutritional vitamin K deficiency, however, the hepatic menaquinone concentration remained unchanged. Nevertheless, an oral dose of MK-9 rapidly restored prothrombin concentration to normal (see also Figure 2). This finding suggests that the menaquinones in liver (and possibly in other tissues as well) may not be readily recruited from tissue stores to serve as cofactors for γ-glutamylcarboxylase. Another interest-

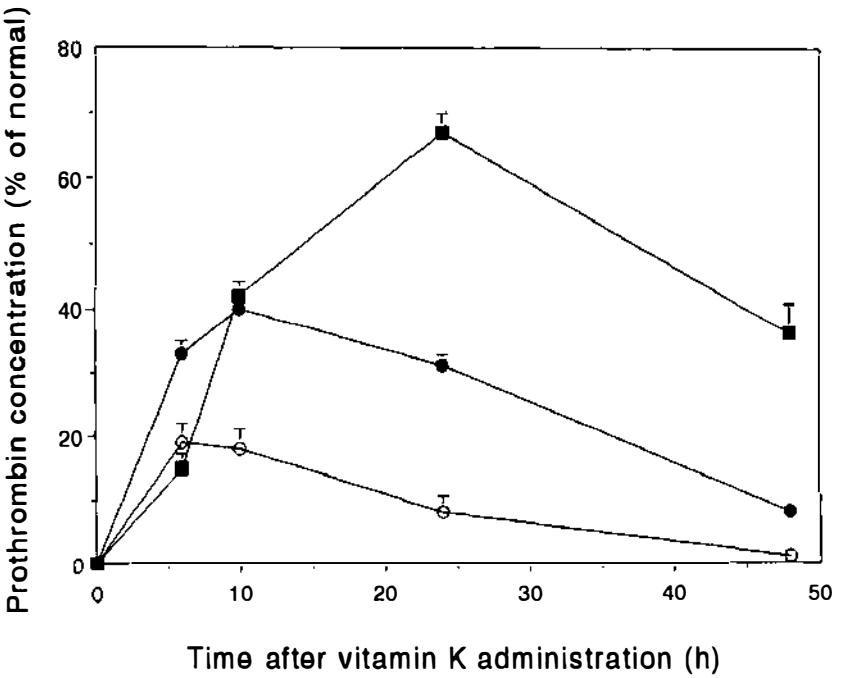

Figure 2 Absorption of various forms of vitamin K in the vitamin K–deficient rat. Rats were made vitamin K deficient by feeding them a vitamin K–deficient diet (Hope Farms, Woerden, The Netherlands) and by using anal cups for the duration of the experiment. Doses of 10 nmol of either phylloquinone (*solid circle*), MK-4 (*open circle*), or MK-9 (*solid square*) were administered orally. Prothrombin concentration was measured in a one-stage clotting assay and is expressed as a percentage of normal pooled rat plasma. Points are means of six animals ± standard error (SE).

ing observation is that phylloquinone counteracts the effect of warfarin in liver but not in bone (76). If this phenomenon could be explained by the preferential use of higher menaquinones for the synthesis of bone Gla proteins by osteoblasts, then the oral administration of MK-9, rather than phylloquinone, would neutralize the effect of warfarin in bone.

From the above data we conclude that measurement of the total vitamin K content of isolated food items is insufficient to determine the amount of bioavailable vitamin K and to assess the recommended daily allowance (RDA). Other factors that should be considered in nutritional research are the form in which the vitamin is ingested (membrane bound or solubilized in fat), the effects of other components of the diet, and the role of bile secretion. In addition to measuring the total amount of vitamin K in various food items, investigators should determine its bioavailable fraction in each of these items under standard conditions in human volunteers.

ASSESSMENT OF VITAMIN K STATUS

Ideally, a variable may serve as a marker for vitamin K status if (*a*) it is correlated with the respective tissue concentrations (liver, bone) at any time; and (*b*) it reflects a person's total bioavailable vitamin K reserves (phylloquinone + menaquinones). Here we discuss to what extent a number of commonly used markers meet these requirements.

Plasma Phylloquinone Levels

Although the reliable determination of phylloquinone in plasma involves an elaborate procedure, several specialized research laboratories have successfully determined phylloquinone for the past 5–10 years (30, 83). At this time, however, which factors determine plasma phylloquinone levels, and which plasma phylloquinone levels correspond to tissue vitamin K sufficiency or deficiency, remains unclear. Figure 1 clearly shows that plasma phylloquinone levels are affected by the type of food consumed during the hours prior to blood sampling. For this reason, most workers in the field use fasting samples to quantify vitamin K. After enteral absorption, phylloquinone is incorporated into chylomicrons for transportation to the liver and other target tissues in close association with triglycerides and lipoproteins. In a large survey of a normal population, plasma phylloquinone and triglyceride concentrations were well correlated (83). The extremely low phylloquinone levels in newborn babies may thus be partly explained by the very low plasma lipoprotein levels present at birth and may have led to an underestimate of the vitamin K stores in tissues. An explanation for these low levels was put forth by Shearer (89). Both Sadowski et al and Shearer suggested that the ratio of plasma phylloquinone to plasma triglycerides would be a better index of vitamin K status than plasma phylloquinone alone (83, 90). Patients with various lipid disorders, especially those associated with hypertriglyceridemia, had very high plasma phylloquinone levels (85, 89). Whether tissue vitamin K concentrations are elevated in these patients as well remains to be determined.

Plasma phylloquinone concentrations were also reported to be genetically influenced by the apolipoprotein E genotype, an important determinant of the chylomicron remnant clearance rate. The plasma phylloquinone concentration in homozygous carriers of the variant E2 was apparently higher than that in carriers of E3 and was lowest in carriers of E4 (85) because apolipoprotein E2 facilitates vitamin K–carrying chylomicron remnant uptake by the liver to a lesser extent than do the variants E3 and E4. Thus, remnants containing E2 remain in the circulation for a longer period of time, resulting in higher plasma vitamin K concentrations. In the latter E2 variants, increased levels of undercarboxylated bone Gla proteins were observed, indicating marginal vitamin K

status. From these data we conclude that plasma triglyceride levels are an important determinant of circulating phylloquinone levels. Only in a limited number of cases (e.g. in hospitalized patients with poor nutritional status) are low phylloquinone levels potentially indicative of impaired vitamin K reserves (90). Realizing the limitations of using plasma phylloquinone as a marker for vitamin K status, investigators sought other markers. For example, Knapen et al proposed that a person should be considered vitamin K sufficient if all known Gla proteins occur in a fully carboxylated form (43). According to this definition, vitamin K deficiency might be regarded as a state in which at least one of the Gla proteins occurs in an undercarboxylated form.

Descarboxy Proteins

Well-defined Gla proteins play a role in two physiological processes: blood coagulation and bone metabolism. The vast majority of circulating Gla proteins are involved in blood coagulation and are synthesized by hepatocytes. Traditionally, descarboxy-prothrombin has been used as a marker to detect vitamin K deficiency. Several tests for descarboxy-prothrombin have been described, the most sensitive of which is based on monoclonal antibodies that specifically recognize descarboxy-prothrombin (102).

Nutritional vitamin K intake has been shown to decrease with age (see below), and low serum levels of both phylloquinone and menaquinones have been reported in elderly persons (30, 39, 83). This loss does not appear to affect blood coagulation factors, however. Osteocalcin, on the other hand, seems to be more sensitive to low vitamin K intake. A number of clinical trials showed that circulating descarboxy-osteocalcin is common in healthy postmenopausal women and disappears when vitamin K supplementation is initiated (42, 68, 69, 94). Even in healthy newborns, whose vitamin K status is known to be precarious, no or very low levels of descarboxy-prothrombin are detectable. In contrast, all babies tested exhibited high concentrations of serum descarboxy-osteocalcin. Moreover, vitamin K supplementation during pregnancy significantly decreased descarboxy-prothrombin levels in the cord sera (40).

These data demonstrate that circulating osteocalcin is the most sensitive known marker for vitamin K status. The difference between the vitamin K–dependent coagulation factors (all synthesized in the liver) and the bone Gla protein osteocalcin suggests that different tissues (at least bone and liver) may have different vitamin K requirements; hence bone tissue may be more prone to vitamin K deficiency than liver. If this is the case, impaired synthesis of some vitamin K–dependent proteins may be far more prevalent in the human population than coagulation assays indicate (79), potentially resulting in an increase in RDAs for vitamin K, notably for the elderly.

GLA PROTEINS OCCURRING IN BONE

Differences and Similarities

Three Gla proteins have been identified in bone tissue: osteocalcin (also known as bone Gla protein, or BGP), matrix Gla protein (MGP), and protein S (31, 54, 71, 73). All three are synthesized by osteoblasts, but only osteocalcin is synthesized exclusively by osteoblasts and odontoblasts (33). Protein S is also produced by hepatocytes, megakaryocytes, and endothelial cells (17, 18), whereas MGP is expressed in most soft tissues (21). Although the three Gla proteins found in bone represent approximately 80% of our body store of Gla, their function in bone remains unclear.

Osteocalcin

In vitro, osteocalcin binds strongly to hydroxyapatite crystals and is a potent inhibitor of hydroxyapatite formation (71). In bone, osteocalcin occurs in close association with the mineralized matrix, to which it is bound via the α-helical Gla domain (32, 33). The carboxy-terminal part of osteocalcin, on the other hand, reportedly possesses chemo-attractant activity for osteoclast progenitor cells involved in bone resorption (49, 50). These and other properties indicate that osteocalcin plays a regulatory role in the mineralization and remodeling of bone tissue (33).

From the concentration of osteocalcin in blood and bone, and from its estimated half-life in both tissues, we can calculate that approximately 20% of the newly synthesized osteocalcin is not bound to the hydroxyapatite matrix in bone, but is set free in the bloodstream. Circulating osteocalcin levels have been shown to correlate with bone formation, as assessed by bone growth and high bone turnover (33). Since osteocalcin is uniquely produced by osteoblasts in bone, its serum level is frequently used as a specific marker for osteoblast activity or bone formation (14, 63). The diagnostic value of serum osteocalcin in addition to other bone markers remains to be determined (63).

Matrix Gla Protein

Despite its high percentage of hydrophilic amino acid residues and its small size (9.6 kDa), MGP is virtually insoluble in aqueous solutions (73). Its affinity for demineralized bone matrix and nonmineralized cartilage suggests interactions with other organic constituents of bone, such as collagen, proteoglycans, or glycosaminoglycans. In bone, mRNA levels coding for MGP are much lower than in kidney, lung, liver, spleen, and vessel wall (21); nevertheless, only trace amounts of immunoreactive MGP could be identified in the various soft tissues, suggesting that most of the secreted MGP enters the circulation. On the other hand, MGP is abundantly present in bone and cartilage (21, 27),

but its function remains a matter of speculation. Owing to its insolubility, most of the protein may aggregate and precipitate following cellular secretion to form part of the extracellular matrix involved in cell adhesion (53). It has been postulated that in soft tissues, MGP may help clear extracellular calcium and protect against tissue calcification (21, 53). On the other hand, the high concentrations of MGP found in bone and cartilage may create conditions that predispose to tissue calcification.

Protein S

Protein S is a single-chain molecule containing 11 Gla residues that plays an inhibitory role in blood coagulation by acting as a cofactor for activated protein C (13). Protein S bears a close structural resemblance to the other vitamin K–dependent proteins of the clotting system, but unlike these proteins, it is not the proenzyme of a serine protease. Approximately 50% of circulating protein S occurs in a free form, and the remainder is bound as a bimolecular complex to C4b-binding protein, a protein involved in the regulation of the complement system (34). Only free protein S is active as a cofactor for protein C; however, its function in the C4b-binding protein–protein S complex has not yet been established. Several observations indicate that inherited protein S deficiency is associated with an increased risk of recurrent thrombosis (16). In 1990 Pan et al reported that two children with well-established protein S deficiency suffered from severe osteopenia and had reduced bone mineral density and (in one case) vertebral body compression fractures (62). This finding suggests an additional role of protein S in bone metabolism and was recently supported by the discovery that protein S is also synthesized by osteoblasts and forms a constituent of the organic bone matrix (54).

Structural Characteristics

With respect to biosynthesis, the three Gla proteins found in bone share a number of characteristics with other Gla proteins, such as the blood coagulation factors. All three are secretory proteins, the intracellular precursor form of which possesses a "leader" sequence. The leader contains a hydrophobic "pre" sequence required for translocation across the endoplasmic reticulum. Moreover, all three contain an 18-residue "pro" sequence that serves as a recognition signal for the vitamin K–dependent carboxylase. (For extensive reviews on vitamin K–dependent carboxylase see 24, 59, 93, and 101.) Whereas in all other known Gla proteins the pro sequence is located directly in front of the amino terminus of the mature protein, MGP has an internal carboxylase recognition sequence that is not removed during protein maturation. In MGP, Glu residues on both sides of the recognition sequence are converted into Gla. Structural elements required for recognition by carboxylase are thought to be the highly conserved amino acid residues at the positions –16 (Phe) and –10

(Ala/Gly) (97). The Gla domain itself also contains a sequence found in all mammalian Gla proteins known to date: Gla-X-X-X-Gla-X-Cys (78). However, its relevance for the enzyme-substrate interaction is not clear at this time.

In humans, at least two of the bone Gla proteins were shown to occur in an undercarboxylated form, notably at positions 17 for osteocalcin and 2 for MGP (74, 75). These observations were reported by only one group and need confirmation, notably in bone samples from subjects at different ages. Conversely, undercarboxylation of the hepatic Gla proteins is rarely seen in healthy subjects. The occurrence of undercarboxylated Gla proteins indicates a (biochemical) vitamin K deficiency, which is consistent with a poor vitamin K status of human bone tissue. The Gla content of circulating osteocalcin has been proposed as an exceptionally sensitive and useful indicator of in vivo vitamin K status. Moreover, the function of osteocalcin in human bone can seemingly be compromised by nutritional levels of vitamin K that can still support a normal blood coagulation time (77).

Vitamin K Antagonists

A state very similar to vitamin K deficiency results from the administration of 4-hydroxycoumarins, which act as vitamin K antagonists. Via a blockade of the enzyme vitamin K–epoxide reductase, these compounds prevent recycling of vitamin K into the hydroquinone, the active cofactor for carboxylase (100). The negative effects of coumarins on bone development were first noticed in babies of women who had used these drugs as oral anticoagulants during the first semester of pregnancy. These children frequently exhibited bone defects caused by excessive calcification of the epiphyses and had irregular growth of the facial and long bones (66). Similar clinical features were reported in an infant with biochemical evidence of a congenital deficiency of vitamin K–epoxide reductase (65). These observations suggest that—at least in young, rapidly growing bone—Gla proteins have an important regulatory function and help prevent irregular precipitation of calcium salts. Similar effects could be provoked in young rats, in which prolonged warfarin treatment resulted in complete closure of the growth plates and cessation of femoral growth (72). Remarkably, adult bone seems to be more resistant to the effect of coumarin derivatives, which are now widely used as oral anticoagulants to prevent thrombosis and myocardial (re-)infarction.

VITAMIN K DEFICIENCY IN ANIMAL MODEL SYSTEMS

In order to assess dietary influences on vitamin K status and to investigate intestinal absorption, transport, and bioavailability of various forms of purified vitamin K, vitamin K–deficient experimental animals must be available. Traditionally, the rat has been the animal of choice for laboratory investigations.

This section provides a simple protocol for generating vitamin K deficiency in rats.

Prevention of Coprophagy

Under standard laboratory conditions, rats eat 60–80% of their feces (4), and investigators do not always realize how difficult it is to prevent coprophagy in these animals. Because feces contain substantial amounts of menaquinones, they are a rich dietary source of vitamin K. The production of menaquinones may be reduced by inhibiting colonic bacterial growth, either with antibiotics (52) or by mixing the normal diet with cooked white rice in a 1:1 ratio (56). Some antibiotics, however, also have a direct inhibitory effect on the vitamin K–dependent system in the liver, which makes the data difficult to interpret (52). On the other hand, in addition to causing vitamin K deficiency, the white-rice diet may result in other nutritional deficiencies. Many investigators hope that placing the rats in cages with wire-mesh bottoms or in metabolic cages will prevent coprophagy. However, Barnes et al showed that rats maintained on raised wire screens can still ingest 50–65% of the excreted feces (4). This problem can be circumvented in one of two ways. First, one may use germ-free rats (95). Although this procedure has raised no fundamental objections, maintaining the animals under sterile conditions presents a practical problem, especially if the experiments extend over long periods of time and repeated handling of the animals is required. Alternatively, one may use anal cups to collect the feces (4). This simple technique eliminates coprophagy and (in combination with a vitamin K–deficient diet) rapidly induces severe hypoprothrombinemia (5). In our experience, reliable experiments with vitamin K–deficient rats cannot be performed unless these anal cups are used.

Vitamin K–Deficient Diets

To fully induce vitamin K deficiency in rats, they must be fed a diet devoid of vitamin K. In 1959 two such diets were described (5, 55). In most cases, these diets substantially prolonged prothrombin times. However, in subsequent years results with such diets varied, and irrespective of how long the diet was sustained, the rats never exhibited a bleeding tendency, probably owing to incomplete removal of the last traces of vitamin K from the food. Moreover, we found that powdered food may be hygroscopic, and if such food is kept at room temperature for more than 1 week, menaquinone-producing bacteria may develop and thus reduce the effectiveness of the diet. Commercial foods with a very low vitamin K content are now available. In combination with anal cups, this food generally leads to severe vitamin K deficiency, with clotting factor concentrations 2–5% of the starting values within 5–7 days. In these rats, the intestinal flora remains intact and contains normal amounts of menaquinones, demonstrating once more the poor absorption of menaquinones

in the colon (95). As an example of the usefulness of this experimental animal model, we have depicted the absorption and bioavailability of a single dose of three different forms of vitamin K in rats made vitamin K deficient according to the above protocol (see Figure 2).

The Warfarin + Vitamin K Regime

Because hemostasis is the first physiological system in which vitamin K deficiency results in a physical impairment, it is difficult to investigate long-term effects of a poor vitamin K status on other systems, such as calcium metabolism. In one study, a reduced vitamin K intake was associated with the development of hypercalciuria, but unfortunately the method of generating vitamin K deficiency was inadequate and insufficiently standardized (81). Nevertheless, the conclusions reached are consistent with other, mainly clinical, data presented below.

Study of the role of Gla proteins in bone was greatly facilitated by Price & Kaneda (76), who showed that (*a*) oral anticoagulants inhibit the carboxylation of Gla proteins both in liver and in bone; and (*b*) vitamin K antagonizes this effect in liver but not in bone. Hence, protocols were developed based on high doses of warfarin combined with a certain amount of vitamin K, which led to a normal hemostasis and, in parallel, to a complete blockade of the carboxylation of bone Gla proteins. The model has been firmly established both in rats and in lambs (64, 76). Remarkably, bone abnormalities become obvious in rats only after several months, and in other tissues, this warfarin + vitamin K regimen had no apparent effect. The reported effects of warfarin on bone in lambs were more prominent and included osteopenia with a 30% lower bone mass in 3 months (relative to control animals), mildly decreased bone resorption, strongly decreased bone formation, irregular calcium deposition, and remodeling abnormalities.

CLINICAL INVESTIGATIONS

Vitamin K Status in Humans

The nutritional intake of vitamin K has been shown to decrease with age; during the seventh decade, this intake decreases by 50% (Figure 3). Circulating vitamin K also decreases with age (37, 83) and is significantly lower in osteoporotics and subjects who sustained a hip fracture than in age-matched controls (30, 39). In the bone tissue itself, vitamin K concentrations are strongly reduced in hip fracture patients (38). The key question is whether the poor vitamin K status in elderly subjects is an independent risk factor for osteoporosis or if it merely reflects a poor nutritional state in this group.

As explained above, descarboxy proteins are the most reliable markers for vitamin K status of the tissues in which they are produced. Prothrombin is the

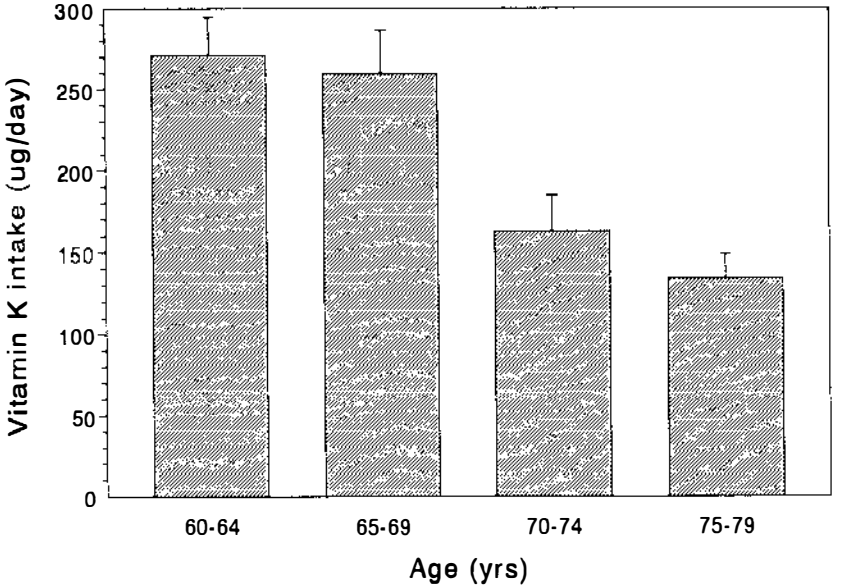

Figure 3 Dietary phylloquinone intake as a function of age. The data were obtained from apparently healthy postmenopausal women (20 per age group) using an elaborate food frequency questionnaire and from the published list of Booth et al (7). Error bars indicate SE.

usual marker for liver, and several techniques can be used to detect descarboxy-prothrombin in blood plasma. The degree of carboxylation of circulating osteocalcin is generally determined using an indirect technique, i.e. by measuring its affinity for hydroxyapatite (hydroxyapatite binding capacity, or HBC) (42, 76). Whereas the circulating immunoreactive osteocalcin (irOC) is a marker for bone formation, HBC is a marker for vitamin K status of bone. Both tests need to be improved, however. Several commercial osteocalcin test kits are available, but the measured osteocalcin levels vary considerably; this variation is probably related to the specificity of the antibodies used (70). Therefore, data on irOC obtained with different kits are not easily comparable. It has been demonstrated that HBC is decreased during vitamin K deficiency in newborns and during coumarin treatment in adults (40, 99), but because no direct test exists for descarboxy-osteocalcin, the possibility that osteocalcin degradation products contribute in an unpredictable way to HBC values cannot be ruled out. Here we consider changes in HBC as a result of changes in vitamin K status of the bone.

Circulating descarboxy-prothrombin is rarely seen in adults, including elderly subjects. It then follows from the above discussion of descarboxy proteins that liver must be vitamin K sufficient in the vast majority of the elderly

population. In two successive trials among postmenopausal women, however, Knapen et al found HBC values to be well below premenopausal values (42, 44). Others reported similar observations (68, 94). It was concluded that elevated levels of circulating descarboxy-osteocalcin are common in elderly women. Moreover, the occurrence of undercarboxylated serum osteocalcin was found to be a strong predictor of subsequent hip fracture (94).

Vitamin K Intervention Studies

To date, several prospective intervention studies with vitamin K have been published in the literature. Vitamin K supplementation (Konakion®, 1 mg/day) has been shown to significantly increase the low HBC levels often found in postmenopausal women (42, 44, 68). In addition, several well-known markers for bone formation (irOC and alkaline phosphatase) increased after vitamin K treatment (42, 44, 60, 68). Markers for bone resorption (fasting urinary calcium and hydroxyproline excretion) decreased with vitamin K treatment, but only in those individuals with a high initial calcium loss. The effect of vitamin K on loss of bone mass was measured in a number of Japanese studies (2, 60). In hemodialysis patients as well as in osteoporotic women, MK-4 (Menatetrenone®, 45 mg/day) reportedly caused a substantial reduction in rate of bone loss and, in a number of cases, even resulted in a 1–2% increase of bone mass over one year. However, because the Japanese studies have not been confirmed in Caucasian women, whether the data on bone mass may be extrapolated to this population remains unclear. Contributing to this uncertainty is the large difference in nutritional habits between Japanese and Caucasian women, notably in terms of calcium intake. Since milk consumption is very low in China and Japan, the mean calcium intake in these countries is less than 300 mg/day (23, 47). If vitamin K merely stimulated intestinal calcium absorption, this effect would obviously have a far greater impact on bone metabolism in Japanese than in Caucasian women.

Effects of Coumarin Treatment

The finding that the action of coumarin derivatives in liver is opposite to that of vitamin K seemed to warrant an investigation of the effect of these drugs on bone and bone markers in patients undergoing long-term oral anticoagulant treatment. Unfortunately, the amount of conclusive data is very limited at this time. All publications are cross-sectional studies in which patient groups are compared with healthy control groups. The outcome of these studies is therefore strongly influenced by the extent to which the patient and control groups are comparable, which may account for the contradicting results. In three studies, increased urinary calcium loss and reduced bone mass were reported after long-term anticoagulant treatment (20, 41, 80), but other studies found no such effect (67, 82). Circulating irOC remained constant (20, 57) or was

decreased (99), whereas HBC was strongly decreased in all cases where these values were measured (41, 57). No data are available on other markers for bone metabolism, such as alkaline phosphatase and hydroxyproline. The results can likely be improved by stratifying the participants for age and gender, but only one study classified patients in this way (41).

EFFECTS OF VITAMIN K IN BONE CELLS AND ANIMAL SYSTEMS

The results of clinical studies indicating that vitamin K stimulates bone formation and reduces bone resorption are supported by a substantial amount of data obtained from experimental model systems. Using a human osteoblast-like osteosarcoma cell line and a murine osteoblastic cell line, investigators demonstrated that addition of MK-4 to the culture media resulted in suppression of cell proliferation and a simultaneous increase of bone-forming activity, as measured by the cellular excretion of alkaline phosphatase (1) and osteocalcin and by enhanced mineral deposition (45). In those cases in which warfarin was added to the culture media, it was found that, like in liver, this compound antagonized vitamin K action. In osteoblast-like cells, MK-4 also substantially reduced prostaglandin E_2 synthesis via inhibition of the osteoblastic enzyme prostaglandin H synthase (46). Prostaglandin E_2 is a potent bone-resorbing agent, which may partly explain the vitamin K–induced decrease of bone loss in vivo. Using the mouse calvaria culture system, Hara et al demonstrated that MK-4 also inhibits bone resorption in a second, prostaglandin E_2–independent way (28).

These data are consistent with two series of experiments in rats. In the first investigation, the animals were ovariectomized to induce the rapid bone loss resulting from estrogen depletion, and vitamin K was found to inhibit the decrease of bone density, bone mineral content, bone strength, and bone hydroxyproline content (3). In the second investigation, rats were subjected to corticosteroid treatment (prednisolone), which induces rapid loss of bone mass. In this case, vitamin K appeared to antagonize the prednisolone-induced losses of bone density, bone strength, and bone calcium content. On the basis of the biochemical markers, the authors concluded that the net result of vitamin K action was brought about by a stimulation of bone formation and by a reduction of bone resorption (29).

SUMMARY AND PERSPECTIVES

Vitamin K in Food

Although the importance of nutritional phylloquinone intake remains unquestioned, increasing evidence suggests that menaquinones also contribute to

human vitamin K status. Several independent observations argue against a major role for menaquinones formed by the intestinal flora, but the importance of nutritional menaquinones seems to have been underestimated until now. If taken orally, menaquinones are absorbed in the gastrointestinal tract, but our knowledge of their distribution in various foods and of their plasma transport and tissue distribution is limited. Another point of interest is the association in which the K vitamers are present in the diet and how this influences their intestinal absorption and relative contribution to human vitamin K status. We feel that the importance of membrane-bound forms of vitamin K (both in green vegetables and in the colon) is currently overestimated. Solubilized forms of vitamin K, such as those found in dairy produce, may prove to be better absorbed and thus satisfy a substantial part of the human daily vitamin K requirement. The fraction of vitamin K extracted from the diet, however, is probably less than generally expected. This finding explains why a relatively mild reduction of nutritional vitamin K intake led to a significant decrease of urinary Gla excretion within three days (19). Both dietary vitamin K intake and intestinal absorption of fat-soluble vitamins in general decrease with age, which may partly account for the prevalence among the elderly of a biochemical vitamin K deficiency that manifests itself by undercarboxylation of the bone Gla proteins. One must determine whether the different K vitamers accumulate in all tissues at a constant ratio or whether they exhibit a certain tissue specificity based on their hydrophobicity or on tissue lipoprotein receptors, for example.

Vitamin K Deficiency

Vitamin K deficiency must be redefined, not on the basis of impaired blood coagulation tests but on the basis of undercarboxylation of Gla proteins. As vitamin K intake decreases, circulating osteocalcin seems to be the first Gla protein to occur in an undercarboxylated form; we therefore consider it the most sensitive marker for vitamin K status at present. The conclusion that poor vitamin K status is far more prevalent than previously assumed will likely be the subject of increasing interest. In this respect, the relation between vitamin K status and various aspects of calcium metabolism (e.g. maintenance of bone mass, urinary calcium loss, vascular mineralization) is of potential importance.

Bone Metabolism and Vascular Calcification

Accumulating evidence suggests that atherosclerotic vessel wall mineralization must be considered an actively regulated process involving various cell types (15). Several recent findings support the view that the mineralization process in the atherosclerotic plaque may be similar to that in bone. Occasionally, artery wall calcifications are structurally identical to bone tissue and may consist of trabecular bone and include cellular elements resembling hematopoietic bone marrow. Biochemical and X-ray diffraction analysis have dem-

onstrated that the mineral phase consists of hydroxyapatite (6). A potent osteoblastic differentiation factor, bone morphogenetic protein 2-a, is expressed in the calcified plaque. This protein was shown to be produced by cells cultured from aortic vessel wall that also formed calcified nodules similar to those found in bone cell cultures (9). Several bone-associated proteins with a high affinity for hydroxyapatite have been detected in the atherosclerotic plaque and seem to be associated with the calcified areas thereof (86). These proteins include osteopontin (15), osteonectin (87), and the Gla proteins osteocalcin (48, 86) and MGP (87). Of special interest is the observation that in the plaque, MGP is locally produced by smooth muscle cells and macrophages and is associated with matrix vesicles similar to those found in developing bone. It is tempting to speculate that certain cells present in the vessel wall may differentiate to osteoblast-like cells that actively participate in a local mineralization process. Recently, a subpopulation of cells with osteoblast-like properties was isolated from the human intimal vessel wall. These cells could secrete alkaline phosphatase, osteocalcin, osteonectin, osteopontin, and collagen type I in vitro and form multicellular nodules with concomitant hydroxyapatite formation (101). The role of Gla proteins in this process may be comparable to that in bone, and vitamin K status could also conceivably affect vascular mineralization. This hypothesis is consistent with recent data from a population-based study, in which low nutritional vitamin K intake and low HBC values were shown to be associated with the presence of atherosclerotic calcifications in the abdominal aorta (K-SG Jie, ML Bots, C Vermeer, JCM Witteman & DE Grobbee, unpublished data). Additionally, in atherosclerotic women, vitamin K status is correlated with bone mass, suggesting that poor vitamin K status is a common denominator of osteoporosis and atherosclerosis.

A Hypothetical Model

Although a high nutritional vitamin K intake seems to have a positive effect on bone formation, it is associated with reduced calcification of the abdominal aorta. How can we explain why vitamin K promotes calcium deposition in one place yet prevents mineralization at another? Here we put forward a hypothesis based on different functions of two bone Gla proteins: osteocalcin and MGP. During bone development, MGP appears much earlier than osteocalcin, predominantly in the nonmineralized parts of bone and cartilage (61). If vitamin K is antagonized during early embryonic development, excessive mineralization of the (cartilageous) growth plates occurs. We propose that MGP restricts calcium deposition and that it exerts this function in bone and other tissues. Osteocalcin, on the other hand, appears during a later stage of bone development and is mainly associated with the inorganic matrix of bone. Therefore, it may play a role in the accumulation of bone mass. This function may be stimulated by vitamin K administration to subjects with marginal vitamin K status.

The high concentrations of immunoreactive MGP observed in calcified atherosclerotic plaques may be related to or resemble the previously described plaque Gla protein (25). The Gla content of this vascular MGP has not been determined, however. We suggest that MGP is produced by the vessel wall as a defense mechanism against mineralization. In the case of insufficient vitamin K supply to the vessel wall, undercarboxylated—and presumably inactive—MGP will be synthesized. Therefore, vitamin K administration to these subjects might improve their defense mechanism against calcification of atherosclerotic plaques. Further investigations are required to establish the role of bone proteins in plaque formation (8, 36, 87) and the putative link between osteoporosis and atherosclerosis (22).

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