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ROLE OF VITAMIN-K-DEPENDENT PROTEINS IN BONE METABOLISM

Paul A. Price

Department of Biology, B-022, University of California, San Diego, La Jolla, California 92093

CONTENTS

INTRODUCTION

This review discusses (*a*) the structure and possible functions of two vitamin-K-dependent bone proteins, bone Gla protein (BGP; osteocalcin) and matrix Gla protein (MGP), and (*b*) the insights into the mechanisms of vitamin K action that have been obtained by analyzing these two proteins. BGP and MGP are both regulated by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and their functions are presumed to be linked to the actions of vitamin D on bone. At present, these two proteins are the only well-characterized vitamin-K-dependent vertebrate proteins other than the proteins involved in blood coagulation. Comparison of the amino acid sequences of MGP and BGP with the sequences of coagulation proteins has identified probable structures that target proteins for γ-carboxylation. Comparison of the action of warfarin on

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BGP synthesis and on blood coagulation times has also revealed a marked difference in vitamin K metabolism between liver and bone. The general aspects of BGP structure, regulation, biosynthesis, and function were recently reviewed (41) and are not discussed here.

STRUCTURE AND PROPERTIES OF BGP AND MGP

Mature bone Gla protein is a 49- or 50-residue protein containing three residues of γ-carboxyglutamic acid and one disulfide bond (41, 49). BGP is secreted by osteoblasts (34, 41) and is one of the most abundant noncollagenous bone proteins, accounting for 15–20% of the noncollagenous protein in the bone of most vertebrates (41). BGP is also found in dentine and is secreted by odontoblasts (2). Low levels of BGP can be detected in most soft tissues because it is present in plasma (41), but BGP mRNA has not been detected in any nonmineralized tissue examined to date (J. D. Fraser and P. A. Price, personal observations). In vitro, BGP binds strongly to hydroxyapatite ($K_d \approx 10^{-7}$ M) and is a potent inhibitor of hydroxyapatite formation (44). Both properties are lost when the three γ-carboxyglutamic acid residues in BGP are decarboxylated to glutamic acid (40).

The structure of the initial BGP translation product, prepro rat BGP (Figure 1), can be subdivided into a 23-residue transmembrane signal sequence, a 26-residue propeptide, and the 50-residue mature BGP isolated from rat bone

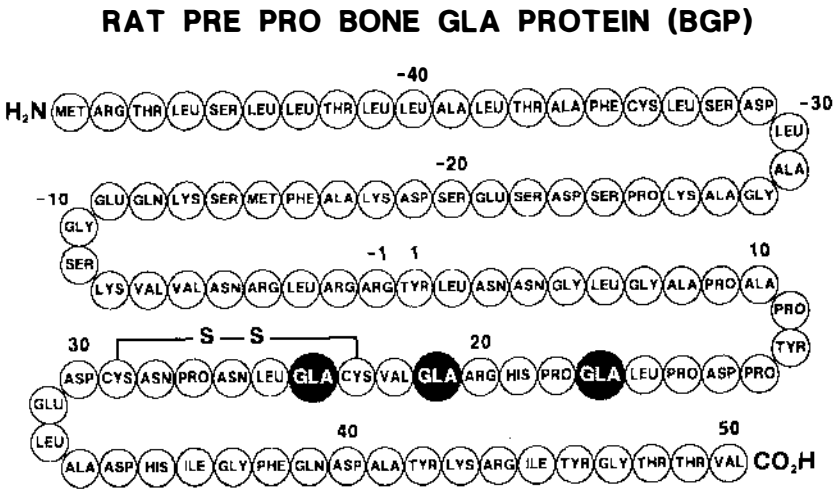

Figure 1 Structure of rat prepro bone Gla protein. The structure of rat prepro BGP was predicted from the rat BGP cDNA sequence (38). N-terminal sequencing of the BGP precursor, which builds up in warfarin-treated osteoblastic cells, established that pro BGP begins at position −26 (lysine) (39). Darkened amino acids denote the three Gla residues.

(38). The structure of pro BGP was established by direct sequence analysis of the BGP precursor that builds up in warfarin-treated rat osteosarcoma cells (39). These studies also showed that the N-terminal sequence of BGP secreted into the culture media of vitamin-K-replete and warfarin-treated cells is identical to mature BGP isolated from rat bone (39), which establishes that proteolytic processing of pro BGP to BGP precedes secretion of BGP from the cell and is independent of γ-carboxylation.

Matrix Gla protein has five Gla residues and is 84 residues in length (Figure 2). There is sufficient sequence identity between BGP and the C-terminal 42 residues of MGP (Figure 3) to indicate that BGP and this region of MGP must have arisen from a common ancestor by gene duplication and subsequent divergent evolution (52). Since bovine MGP is more closely related to swordfish BGP than to bovine BGP (Figure 3), it is likely that the gene duplication event that gave rise to BGP and MGP preceded the divergence of swordfish from other vertebrates, an event that must have occurred early in vertebrate evolution.

In spite of their common ancestry, BGP and MGP have remarkably different physical properties and a different tissue distribution of synthesis. BGP is exceptionally soluble in neutral aqueous solutions (>150 mg/ml). In contrast, MGP is almost insoluble in neutral aqueous solutions (<20 μg/ml) unless denaturants such as 6-M guanidinium HCl are present (37, 51). The insolubility of MGP reflects a strong tendency of the protein to self-associate, which is

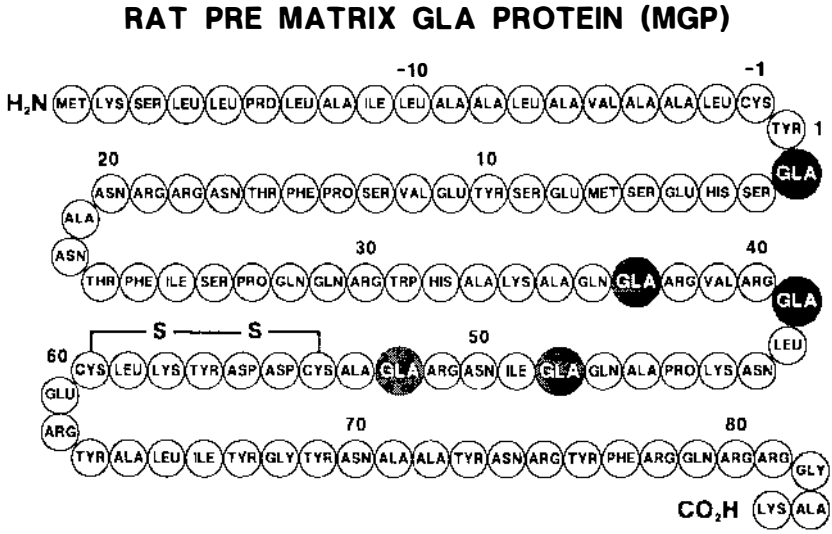

Figure 2 Structure of rat pre matrix Gla protein. The structure of rat pre MGP was predicted from the rat MGP cDNA sequence (45). Darkened amino acids denote the five Gla residues (45, 52).

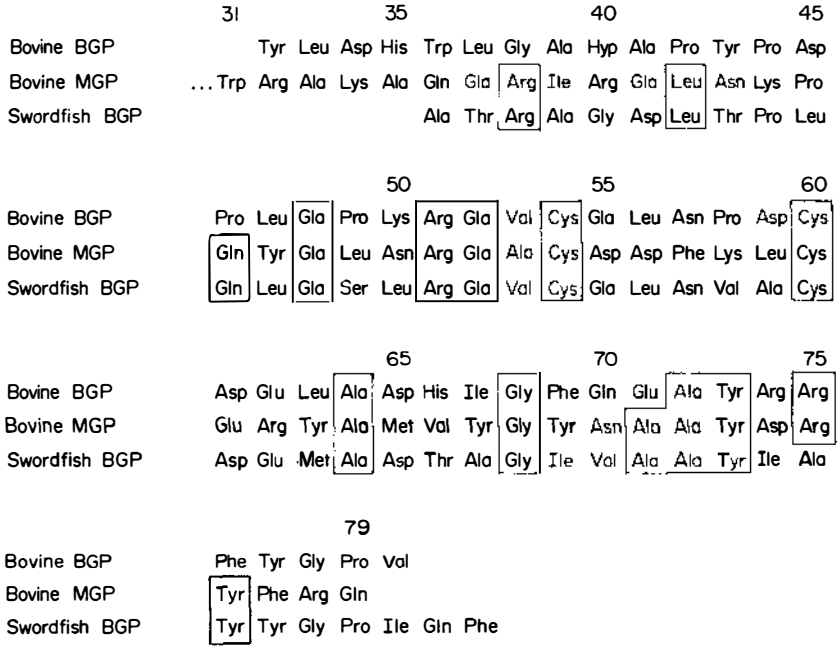

Figure 3 Sequence identity between bovine and swordfish bone Gla proteins and bovine matrix Gla protein. From Price & Williamson (52); used with permission.

particularly noteworthy given its high percentage of hydrophilic amino acids and its small size (52). While BGP is found only in bone and dentine, MGP is found at comparable levels in bone, dentine, and cartilage (18). Northern blot analysis with a cDNA probe for MGP has demonstrated that MGP mRNA is also found in cartilage as well as in bone (18). The high levels of MGP in cartilages that are not mineralized demonstrate that MGP can anchor to the organic matrix independently of mineral. The association of MGP with the organic matrix rather than with mineral was first proposed to account for the observation that a major fraction of MGP emerges only during extraction of demineralized bone with denaturants (37, 51).

We have recently determined the cDNA structure of rat MGP (45). The 521 base pair sequence predicts an 84-residue mature MGP and a 19-residue transmembrane signal sequence. The cDNA structure of MGP reveals it to be the first example of a vitamin-K-dependent protein that lacks a propeptide (45). The absence of a propeptide in MGP demonstrates that the γ-carboxylation and secretion of vitamin-K-dependent proteins need not be linked to the presence of a propeptide or to its proteolytic removal.

The 84-residue rat MGP predicted from the cDNA sequence has an additional five residues at its C-terminus that are not present in the 79-residue

MGP isolated from bovine bone. The first two residues of this C-terminal pentapeptide are Arg-Arg, a dibasic site at which proteolytic processing frequently occurs (24). Although it is not yet known whether proteolytic cleavage to remove the C-terminal pentapeptide precedes the secretion of MGP from the cell, a closely spaced doublet has been noted in the Western blot analysis of MGP in guanidine extracts of bone and cartilage from rat and calf. If this doublet represents the 84-residue protein predicted from the cDNA and the 79-residue protein from bone whose structure was determined by amino acid sequencing, both proteins must be present at comparable levels in the extracellular matrix and proteolytic processing probably occurs after secretion. It is clearly important to know the function of proteolytic processing at the C-terminus of MGP and, in particular, whether the extraordinary water insolubility of the 79-residue MGP isolated from bone, an unusual feature of the protein given its high percentage of hydrophilic amino acids and small size (37, 51, 52), is also a property of the 84-residue protein.

Substrate Recognition By The Vitamin-K-Dependent γ-Glutamyl Carboxylase

We previously showed that the propeptide of BGP contains a region (−16 to −1 in Figure 4) that is homologous to the propeptide of the vitamin-K-dependent proteins involved in blood coagulation, and postulated that the homologous propeptide domain is a recognition site for the γ-carboxylase (38, 39). Although MGP has no propeptide, there is a region of the mature protein (−16 to −1 in Figure 4, BGP numbering) that is homologous to the propeptide of all other vitamin-K-dependent vertebrate proteins.

We have recently discovered a previously unrecognized invariant structure in the Gla-containing region of vitamin-K-dependent vertebrate proteins, the sequence Gla-X-X-X-Gla-X-Cys (E---E-C, positions 17 to 23 in Figure 4) (45). The separation between the first Glu in this invariant structure (E at+17) and the previously recognized invariant Arg in the homologous propeptide domain (R at −1) is 15 to 17 residues in all cases except mouse BGP, where the separation is 12 residues. If the sequences of the homologous propeptide domain and the Gla-containing region of known vitamin-K-dependent vertebrate proteins are compared (−16 to +30, Figure 4), the two vitamin-K-dependent bone proteins, BGP and MGP, are each as closely related to coagulation proteins as to each other. There are two additional residues that are invariant in MGP and in all coagulation proteins but not in BGP (A at −10, E at +30), and one additional residue that is invariant in BGP and in all coagulation proteins but not in MGP (L at +6). There are, for comparison, only two residues that are invariant in BGP and MGP but are not found in coagulation proteins (R at +20, C at +29). These sequence relationships are consistent with the hypothesis that the propeptide–Gla domain unit of known

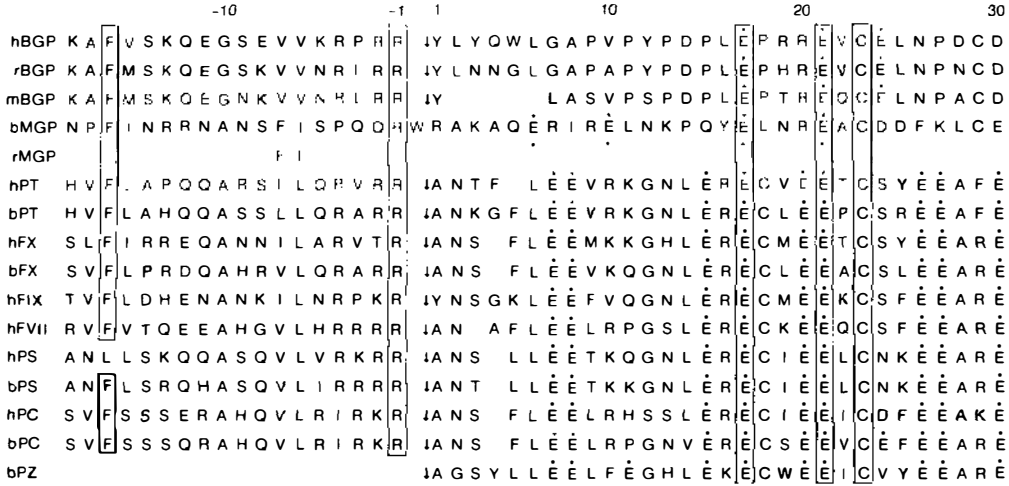

Figure 4 Conserved amino acids in the propeptide and Gla-containing domains of all known vertebrate vitamin-K-dependent proteins (45). Amino acid sequence positions are numbered, with 1 corresponding to the first residue of mature BGP. All other sequences have been aligned to give maximum homology. Arrows preceding residue 1 indicate the site of propeptide cleavage; the absence of an arrow in the MGP structure indicates the absence of a propeptide in this protein. E* refers to γ-carboxyglutamic acid (Gla). Sequences are, from top to bottom, hBGP, human bone Gla protein (3); rBGP, rat BGP (38); mBGP, mouse BGP (3); bMGP, bovine matrix Gla protein (52); rMGP, rat matrix Gla protein (45); hPT, human prothrombin (8); bPT, bovine prothrombin (31); hFX, human factor X(13); bFX, bovine factor X(14); hFIX, human factor IX(23); hFVIII, human factor VII(17); hPS, human protein S (30); bPS, bovine protein S (6); hPC, human protein C (11); bPC, bovine protein C (28); bPZ, bovine protein Z (21).

vitamin-K-dependent vertebrate proteins evolved from a common ancestor by gene duplication and subsequent divergent evolution and indicate that the two bone proteins probably diverged from this common ancestor at about the same time as coagulation proteins.

There is evidence that the invariant propeptide and Gla domain units of vitamin-K-dependent proteins both play a role in substrate recognition by the γ-carboxylase. Site-directed mutagenesis studies have shown that two conserved amino acids in this region of factor IX (F at −16, A at −10; Figure 4) are indeed important to recognition by the γ-carboxylase (22). Although the importance of the E---E-C unit has not yet been tested by site-directed mutagenesis, there is indirect evidence to support a role for the E---E-C unit in γ-carboxylase binding to substrate. A variety of peptides have been synthesized corresponding to the sequences of vitamin-K-dependent proteins in Gla-containing regions but lacking the invariant E---E-C unit (56, 58). None of these peptide substrates have K_m values lower than a few millimolar (7, 61). In contrast, intact BGP and the peptide corresponding to residues 13–29 in prothrombin both prove to be excellent γ-carboxylase substrates (7, 61) after Gla residues are converted to Glu by decarboxylation (40). For both decarboxylated polypeptides, K_m values are 10^2- to 10^3-fold lower than for the best known synthetic peptide substrate, FLEEL (7, 61). Since decarboxylated BGP and prothrombin 13–29 both lack the homologous propeptide domain yet retain the E---E-C invariant structure, the excellent substrate activities of these decarboxylated polypeptides is strong evidence for a role of the E---E-C structure in γ-carboxylase binding to substrate.

A problem with the previously proposed mechanism for substrate recognition by the γ-carboxylase (38, 39) is that the propeptide segment recognized by the γ-carboxylase did not include the glutamic acid substrate. There was, therefore, no mechanism by which the enzyme could directly sense reaction progress and so dissociate from product. If the E---E-C domain is critical to substrate binding, it would provide a mechanism by which γ-carboxyglutamic acid formation and product binding to the γ-carboxylase could be linked, since both glutamic acid residues in this structure are invariably sites of γ-carboxylation (Figure 4). It is noteworthy that a cysteine residue is part of this invariant structure. The γ-carboxylase has an essential sulfhydryl group (58), and the γ-carboxylase assay is carried out under reducing conditions (7, 61) that would be predicted to reduce the disulfide bond in in vitro substrates such as decarboxylated BGP and prothrombin 13–29. We speculate that the invariant substrate cysteine could form a disulfide bond with the essential sulfhydryl of the γ-carboxylase. This covalent bond would form upon substrate recognition, a process that would include interaction with the propeptide and E---E-C domains of substrate, and would serve to anchor substrate to enzyme while γ-carboxylation of accessible glutamic acid residues

proceeds. Upon completion of the γ-carboxylation of glutamic acid residues in the E---E-C unit, altered product binding would then allow disulfide exchange to yield directly the correct disulfide in the protein product (C23 to C29 in BGP and MGP;
enzyme from substrate. One prediction of this model is that synthetic peptides containing the E---E-C structure will prove to be far better substrates for the γ-carboxylase than FLEEL.

The structures of BGP and MGP also provide insight into the factors determining whether given glutamic acid residues in the vicinity of the invariant propeptide and E---E-C units will be fully γ-carboxylated. MGP is the first example of a vitamin-K-dependent protein in which a glutamic acid residue that lies on the N-terminal side of the putative structures involved in substrate recognition is γ-carboxylated. This demonstrates that glutamic acid residues on either side of the substrate recognition site have access to the active site of the γ-carboxylase. The structure of MGP also shows that some glutamic acid residues in the vicinity of the putative substrate recognition site are not γ-carboxylated (residues 5, 8, 11 in Figure 2). This observation indicates that proximity to the substrate recognition site is not sufficient in itself to target glutamic acid residues for modification, and that other factors, such as the secondary structure of the polypeptide or the chemical nature of nearby amino acid side chains, may determine which glutamic acid residues in this region are γ-carboxylated. In our conceptual model of substrate recognition by the γ-carboxylase, enzyme binds target protein at sites that do not directly involve most of the glutamic acid residues subsequently modified. The property determining whether a given glutamic acid residue in the vicinity of the γ-carboxylase becomes modified would therefore be the affinity of that glutamic acid residue for the catalytic site of the γ-carboxylase. Residues with a high affinity for the catalytic site would be quantitatively γ-carboxylated during the lifetime of the complex formed between the γ-carboxylase and the substrate recognition sites (propeptide and E---E-C units). Residues with a very low affinity would not be detectably γ-carboxylated; this presumably is the case for residues 5, 8, and 11 in MGP (Figure 2).

In this model, it is clearly possible to have glutamic acid residues with an intermediate affinity for the catalytic site that are only partially γ-carboxylated during the lifetime of the γ-carboxylase–target protein complex. We believe this is the case for residue 2 in MGP, which is 80% Gla and 20% Glu in the bovine and rat proteins (45, 52), and for residue 17 in human BGP, which is 9% Gla and 91% Glu (41). If partial γ-carboxylation does depend on the affinity of given glutamic acid residues for the catalytic site, sites of partial γ-carboxylation should be sensitive indicators of the probability of γ-carboxylation during the lifetime of the target protein–γ-carboxylase complex. Any factor, such as vitamin K nutritional status or presence of a vitamin

K antagonist, that changes the catalytic rate of γ-carboxylation will, by these arguments, be most readily detected at the glutamic acid residues that are partially γ-carboxylated. For example, if the portion of non-γ-carboxylated glutamic acid residues at a good site is 0.1%, reducing the catalytic rate of the γ-carboxylase by 50% would increase the portion not γ-carboxylated to 0.2%, an amount that cannot be detected by chemical analysis. For residue 2 in MGP, which is normally 20% non-γ-carboxylated, reducing the rate of γ-carboxylation by 50% would increase the portion not γ-carboxylated to 40%, an amount that can be easily detected by chemical analysis. Sites of partial γ-carboxylation in vitamin-K-dependent proteins should therefore be exceptionally sensitive and useful indicators of the in vivo rate of γ-carboxylation.

It is worth noting an obvious corollary to this argument. Proteins with a functionally important glutamic acid residue that is normally a site of partial γ-carboxylation will be far more affected by a given degree of vitamin K deficiency or a given dose of a vitamin K antagonist than will proteins in which all functionally important glutamic acid residues are fully γ-carboxylated. Human BGP is a case in point, and we propose that the function of BGP in human bone can be compromised by nutritional levels of vitamin K that are still able to support a normal blood coagulation time.

THE EFFECT OF WARFARIN ON BGP SYNTHESIS

Before reviewing the BGP response to the vitamin antagonist warfarin, it is necessary to discuss plasma BGP and its relationship to BGP in bone. We developed the radioimmunoassay for BGP, discovered BGP in serum and plasma, and demonstrated that plasma BGP is an excellent marker for bone metabolism (47, 48). Plasma BGP is identical in size to BGP in bone, arises from new synthesis by osteoblasts, and appears to represent the fraction of BGP secreted from the osteoblast that fails to bind to bone mineral and so diffuses into blood (55). These relationships are illustrated schematically in Figure 5.

Shortly after the administration of warfarin to a vitamin-K-replete rat, serum BGP loses its ability to bind hydroxyapatite strongly (Figure 6). This reflects the undercarboxylation of the protein (55), although it is not known whether one, two, or all three Gla residues are in fact required for hydroxyapatite binding. If warfarin is administered daily, bone levels of BGP decrease to 2% of normal over several weeks, essentially at the rate at which bone matrix turns over. Bone levels of BGP remain at this decreased level as long as warfarin treatment is continued (53).

These observations indicate that warfarin affects the γ-carboxylation status of new BGP synthesis soon after its administration. Since normal γ-carboxylation is required for BGP to anchor to hydroxyapatite in bone, nearly

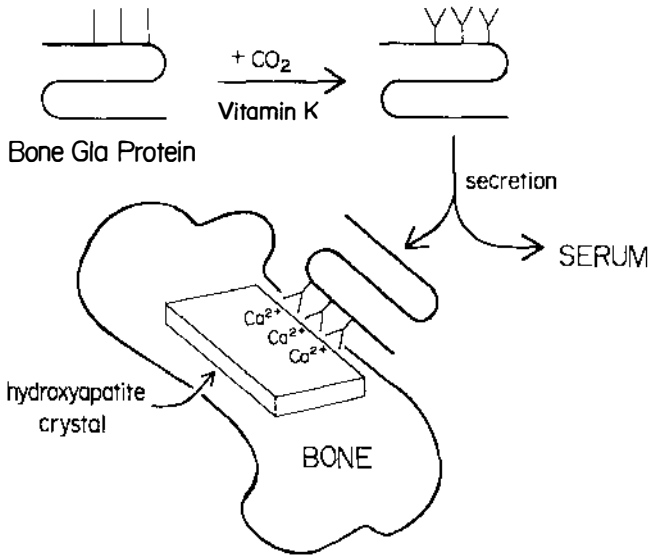

Figure 5 Schematic outline of the probable relationship between bone Gla protein secretion by osteoblasts and its accumulation in bone or serum. In warfarin-treated animals, the non-γ-carboxylated form of BGP that is secreted cannot bind to bone mineral and consequently appears at elevated levels in serum (cf Figure 6).

all new BGP synthesized by osteoblasts escapes to serum, where it elevates total levels of serum BGP (Figure 6). Total bone levels of BGP change slowly, as the BGP-replete matrix synthesized prior to warfarin treatment is gradually resorbed by osteoclasts and replaced with a BGP-deficient matrix.

The protocol we devised to determine the effect of chronic warfarin administration on bone levels of BGP employed massive warfarin dosages (7.7 mg/100 g body weight/day) together with the minimum vitamin K dosage needed to prevent acute problems of bleeding (53). Animals maintained on this protocol for the long periods needed to observe altered bone structure (8 months) were remarkably free of any bleeding problems, even though bone levels of BGP remained at 2% of normal (54).

We have recently discovered that the vitamin K dosage employed in our warfarin protocol completely counteracts the effect of warfarin on blood coagulation after approximately 7 days (46). We had previously failed to recognize this effect because coagulation times were in fact quite elevated at 3 to 4 days of treatment with warfarin plus vitamin K, the time arbitrarily chosen in the earlier studies to establish the proper vitamin K dosage. Pretreatment for 7 days with the vitamin K dosage used in the protocol reduced the degree to which coagulation times were prolonged after 4 days of warfarin treatment (46). The prolonged coagulation time at 4 days of warfarin treatment could be completely prevented by administering a single dose of 40

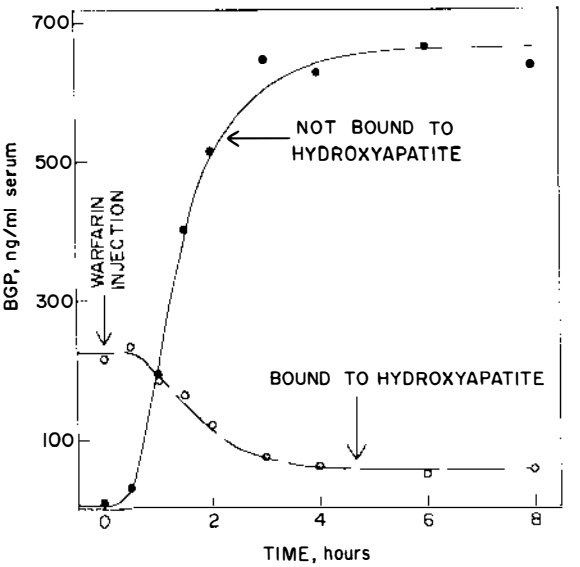

Figure 6 Effect of warfarin on the hydroxyapatite binding properties of serum BGP in 1-month-old rats. At time zero, rats received 7.7 mg of warfarin per 100 g body weight. Serum samples were removed at the indicated times and tested for hydroxyapatite binding as described. Data points represent serum BGP either bound *(open circles)* or not bound *(closed circles)* to hydroxyapatite. [From Price, Williamson & Lothringer (55); used with permission.]

mg of vitamin K per 100 g body weight one day prior to the start of treatment with warfarin plus vitamin K (46). Surprisingly, the hydroxyapatite binding activity of serum BGP remained at 5% of normal regardless of vitamin K pretreatment or the duration of treatment with vitamin K plus warfarin (46).

To obtain a better measure of the ability of vitamin K to counteract the effect of warfarin on blood coagulation and on serum BGP, we evaluated the warfarin dose dependence of both effects in rats treated with vitamin K compared to rats not receiving the vitamin (46). As can be seen in Figures 7 and 8, vitamin K treatment completely prevented the ability of high warfarin doses to prolong blood coagulation times but had essentially no ability to alter the warfarin dose dependence of the serum BGP response. Since, in the absence of vitamin K treatment, comparable warfarin dosages were required to double coagulation times (50 μg/100 g/day) and to reduce the hydroxyapatite binding activity of BGP by 50% (30 μg/100 g/day), the inherent warfarin sensitivities of the bone and liver systems are identical. The difference between the two systems is that vitamin K treatment completely counteracts warfarin effects on blood coagulation but not on BGP synthesis.

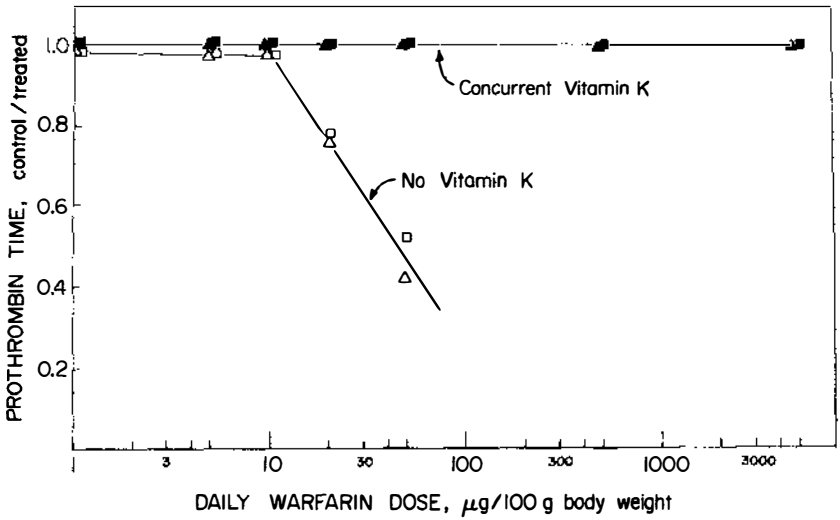

Figure 7 Comparison of the effect of warfarin dosage on the coagulation times of plasma from rats treated with vitamin K_1 to that of rats not receiving vitamin K.

Solid symbols: rats 22 days of age that received daily injections of 0.77 mg vitamin K_1 per 100 g body weight for 6 days and then were given this vitamin K_1 dosage plus daily dosages of the indicated amount of warfarin for 7 days *(solid triangles)* and for 14 days *(solid squares)*.

Open symbols: rats 29 days of age that received daily dosages of the indicated amount of warfarin for 7 days *(open triangles)* and for 14 days *(open squares)*. Each data point is the ratio of the average coagulation time in four untreated controls to the average coagulation time in four animals from each treatment group. [From Price & Kaneda (46); used with permission.]

The ability of high vitamin K dosages to counteract the effect of warfarin on blood coagulation has been known for many years (29). Present evidence indicates that vitamin K treatment enables a warfarin-insensitive enzyme to reduce the vitamin K epoxide product of the γ-carboxylation reaction back to the hydroquinone (62, 63). Without vitamin K treatment, endogenous levels of vitamin K in the hepatocyte are evidently too low to allow significant reduction of the epoxide by this warfarin-insensitive pathway and γ-carboxylation ceases. Our results demonstrate that this warfarin-insensitive pathway is not significantly active in the osteoblasts of vitamin-K-treated rats. Osteoblasts therefore must either lack the warfarin-insensitive epoxide reductase or are unable to accumulate sufficient vitamin K to enable this enzyme to function at a rate sufficient to support γ-carboxylation of BGP.

EFFECTS ON BONE OF LONG-TERM TREATMENT WITH WARFARIN PLUS VITAMIN K

The discovery that vitamin K counteracts the effect of warfarin on blood coagulation but not on BGP synthesis allows us to employ quite large warfarin

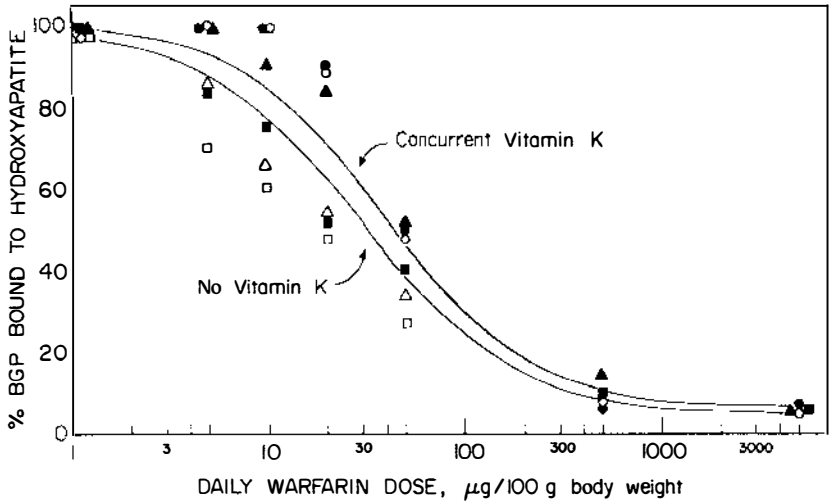

Figure 8 Comparison of the effect of warfarin dosage on the hydroxyapatite binding activity of serum BGP from rats treated concurrently with vitamin K_1 to that of rats that did not receive vitamin K_1.

Solid symbols: rats 22 days of age first received daily injections of 0.77 mg vitamin K_1 per 100 g body weight for 7 days and then were given this vitamin K_1 dosage plus daily dosages of the indicated amount of warfarin for 1 day *(solid circles)*, 7 days *(solid triangles)*, and 14 days *(solid squares)*.

Open symbols: rats 29 days of age that received daily injections of the indicated amount of warfarin for 1 day *(open circles)*, 7 days *(open triangles)*, and 14 days *(open squares)*. Each data point represents the average percentage of serum BGP that bound to hydroxyapatite in four animals from each treatment group. [From Price & Kaneda (46); used with permission.]

doses without affecting blood coagulation (46). Thus the daily dosage of warfarin, 7.7 mg/100 g/day, was over 150-fold greater than the dose required to lower the hydroxyapatite activity of BGP by 50%, but blood coagulation times were never greater than normal. Any physiological changes elicited by the warfarin plus vitamin K protocol must therefore be interpreted as a direct effect of warfarin on bone, or on any other tissue with similar vitamin K metabolism, rather than as any possible effect on blood coagulation.

The general health of rats maintained from birth to 8 months of age on the warfarin plus vitamin K protocol was exceptionally good. Weight gain was unimpaired over the 8 months of treatment (53, 54), although there was a slight decrease in bone growth after 3 months (see below). At no time during the treatment was a significant difference noted in any parameter measured in the standard SMAC test (sequential multiple analyzer with computer) of serum from experimental and control rats. There was also no difference between experimental and control animals in visual appearance, radiological analysis of skeletal morphology, dentition, or the general ability to see and hear.

In normal rats, the only effect of long-term treatment with warfarin plus

vitamin K that can be unambiguously related to the direct action of warfarin is the excessive mineralization of growth plate cartilage, with eventual fusion of the growth plate (54). In rats treated from birth to 8 months of age with warfarin plus vitamin K, growth plates were completely fused and longitudinal growth had ceased. The overall length of the long bones was accordingly reduced; for example, tibias of experimental animals were 7% shorter than the 8-month-old control rats. Subsequent investigations revealed that growth plate mineralization first becomes apparent at 3 months of age, the time at which rapid skeletal growth is complete in the rat. This pattern of excessive growth plate mineralization is strikingly similar to the fetal warfarin syndrome in humans, a disorder seen in infants whose mothers had received warfarin during the first trimester of pregnancy (19).

We have interpreted these observations as evidence that vitamin-K-dependent bone proteins normally inhibit the seeded crystal growth of hydroxyapatite from the fully mineralized metaphysis into growth plate cartilage. When the action of vitamin K is antagonized by warfarin, the under-γ-carboxylated bone proteins now secreted are unable to retard seeded crystal growth, and mineralization consequently engulfs the longitudinal septa of growth cartilage. This eventually causes growth plate fusion. Matrix Gla protein is currently the best candidate for the vitamin-K-dependent protein that normally inhibits growth plate mineralization, since MGP is the only vitamin-K-dependent protein known to be synthesized in cartilage (18). However, because MGP is water insoluble (37, 51), it has not yet been possible to evaluate the ability of MGP actually to inhibit hydroxyapatite crystal growth. It is worth noting that the structurally related protein BGP is a potent inhibitor of hydroxyapatite crystallization only if it is γ-carboxylated (40, 41).

We have observed a second defect in the bone metabolism of animals treated with warfarin plus vitamin K, a defect seen only in the context of physiological stress (50). Rats received sufficient dosages of $1,25\ (OH)_2D_3$ to increase BGP synthesis maximally (42), together with either warfarin plus vitamin K or vitamin K alone. Treatment with $1,25(OH)_2D_3$ led to the accumulation of unmineralized osteoid in the tibial metaphysis, while treatment with $1,25(OH)_2D_3$ plus warfarin prevented this accumulation. We have interpreted these observations as evidence that $1,25(OH)_2D_3$ stimulated the synthesis of a vitamin-K-dependent mineralization inhibitor, which then caused the accumulation of unmineralized osteoid. Treatment with warfarin caused the putative vitamin-K-dependent mineralization inhibitor to be secreted in an inactive, under-γ-carboxylated form, and consequently allowed the mineralization of osteoid (50). We further suggested that the vitamin-K-dependent mineralization inhibitor is BGP, since the synthesis of BGP is stimulated 6- to 10-fold by $1,25(OH)_2D_3$ (42, 43). With the recent discovery that MGP is similarly stimulated 6- to 10-fold by $1,25(OH)_2D_3$ in osteoblastic

cells (12), either MGP or BGP must now be regarded as a candidate for the $1,25(OH)_2D_3$-regulated protein that inhibits osteoid mineralization.

The warfarin treatment protocol has also been used to prepare bone matrix for in vivo and in vitro tests of mineralized bone particle phagocytosis and removal. When implanted in a vitamin-K-replete rat, the ground bone from warfarin-treated rats was removed at only 60% of the rate of ground bone from control rats (26). A similar impairment was also seen in the in vitro degradation of bone particles by monocytes (25). These effects are apparently due to the impaired recruitment and differentiation of cells capable of removing the dead bone particles (15). Although the identity of the recruited cells has yet to be firmly established, these cells do have tartrate-resistant acid phosphatase, a biochemical marker for the osteoclast. Since high concentrations of BGP are chemotactic for monocytes (32), a cell fraction thought to contain osteoclast precursors, it has been suggested that the impaired degradation of warfarin-treated rat bone is due to low levels of BGP.

One interpretation of the observation that dead bone matrix particles from warfarin-treated rats are poorly degraded in vivo and in vitro is that a vitamin-K-dependent protein, thought to be BGP, plays a critical role in the normal resorption of bone (26). The stimulation of BGP synthesis by $1,25(OH)_2D_3$ would then be a mechanism by which $1,25(OH)_2D_3$ accelerates bone resorption. Although this is an intriguing model, the available in vivo data on the effects of warfarin treatment on bone metabolism do not currently support it. Long-term warfarin treatment does not make rats detectably osteopetrotic, the in vivo consequence of the total absence of osteoclastic bone resorption. Furthermore, long-term warfarin treatment has no effect on the ability of rats to resorb bone in response to dietary calcium deficiency (53) or $1,25(OH)_2D_3$ treatment (50).

IMPLICATIONS OF TISSUE DIFFERENCES IN VITAMIN K METABOLISM FOR WARFARIN PROPHYLAXIS

The obvious implication of the discovery that vitamin K counteracts warfarin effects on blood coagulation but not on BGP synthesis is that blood coagulation tests provide a reliable measure only of the effect of warfarin on the hepatocyte. It is erroneous to infer warfarin sensitivity on bone from its effect on coagulation times, because dietary levels of vitamin K (from food and intestinal flora) determine the dose required to prolong blood coagulation but not the dose required to inhibit normal BGP synthesis. Thus it is possible to have two individuals, one with a high average daily intake of vitamin K and a correspondingly high warfarin dose required for prolonged blood coagulation time and another with a low vitamin K intake and a correspondingly far lower warfarin dose required to increase coagulation times. The first individual, based on our results, will have a far greater impairment of BGP synthesis than

the second. This individual will be correspondingly at greater risk for any complication, such as bone metabolic changes noted above, that is attributable to the impaired synthesis of a Gla-containing protein by an extrahepatic tissue whose vitamin K metabolism is similar to bone. When the therapeutic objective is to reduce the activity of the blood coagulation factors synthesized by the liver, it would be advisable to regulate vitamin K intake so as to minimize the warfarin dosage required.

The second, more intriguing, implication to warfarin prophylaxis is that we can now contemplate an entirely new use for this drug. We can, by selectively counteracting the effect of warfarin on blood coagulation with vitamin K, stringently suppress the γ-carboxylation of Gla-containing proteins in tissues with a vitamin K metabolism similar to bone without any concern whatever about bleeding complications. It seems to us probable that most extrahepatic tissues will fall into this category. Storage of vitamin K is required before a tissue can use the alternate, warfarin-insensitive pathway for reduction of vitamin K epoxide to the hydroquinone (29, 62, 63), and liver is the only organ known to store vitamin K (59).

There is at least one area in which we believe there is sufficient evidence to justify experimental tests of the therapeutic efficacy of the warfarin plus vitamin K protocol. Over the past seven years numerous studies have established the synthesis of vitamin-K-dependent blood coagulation factors by endothelial cells (10, 57), monocytes (35, 60), and macrophages (4, 27, 36). Each cell type has the ability, through the action of its endogenous fibrinolytic pathway, to initiate thrombosis. These extrahepatic vitamin-K-dependent coagulation systems each should, in our view, be susceptible to inhibition by the warfarin plus vitamin K protocol. Among the areas where warfarin has documented activity (via a mechanism that could be due to extrahepatic vitamin-K-dependent blood coagulation) are the suppression of tumor metastasis (5, 16, 20, 33, 64) and the suppression of delayed-type hypersensitivity (9). Certain current therapeutic uses of warfarin may also be better managed with warfarin plus vitamin K. For example, venous thrombosis may in many cases be due to endothelial cell activation of blood coagulation by a vitamin-K-dependent mechanism. This could explain the clinical efficacy of warfarin in this disorder at doses below those that prolong blood coagulation times (1), since present evidence suggests that dietary vitamin K could selectively reduce the effective dose of warfarin on the hepatic system. It is my hope and expectation that the warfarin plus vitamin K protocol developed in our laboratory will initiate an exciting new phase in warfarin prophylaxis.

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