## Warfarin Causes Rapid Calcification of the Elastic Lamellae in Rat Arteries and Heart Valves

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*Abstract*—High doses of warfarin cause focal calcification of the elastic lamellae in the media of major arteries and in aortic heart valves in the rat. Aortic calcification was first seen after 2 weeks of warfarin treatment and progressively increased in density at 3, 4, and 5 weeks of treatment. By 5 weeks, the highly focal calcification of major arteries could be seen on radiographs and by visual inspection of the artery. The calcification of arteries induced by warfarin is similar to that seen in the matrix Gla protein (MGP)–deficient mouse, which suggests that warfarin induces artery calcification by inhibiting γ-carboxylation of MGP and thereby inactivating the putative calcification-inhibitory activity of the protein. Warfarin treatment markedly increased the levels of MGP mRNA and protein in calcifying arteries and decreased the level of MGP in serum. Warfarin treatment did not affect bone growth, overall weight gain, or serum calcium and phosphorus levels, and, because of the concurrent administration of vitamin K, prothrombin times and hematocrits were normal. The results indicate that the improved warfarin plus vitamin K treatment protocol developed in this study should provide a useful model to investigate the role of MGP in preventing calcification of arteries and heart valves. (*Arterioscler Thromb Vasc Biol.* 1998;18:1400-1407.)

Key Words: matrix Gla protein ■ warfarin ■ vitamin K ■ artery calcification ■ heart valve calcification

Matrix Gla protein (MGP<sup>1</sup>) is a 10-kDa secreted protein that contains 5 residues of the vitamin K–dependent calciumbinding amino acid,  $\gamma$ -carboxyglutamic acid (Gla).<sup>1,2</sup> MGP was originally discovered in demineralization extracts of bone but is now known to be expressed by a wide variety of tissues and cell types. The rat tissues with the highest levels of MGP mRNA are cartilage, heart, kidney, and lung,<sup>3,4</sup> and cells known to express MGP mRNA include osteoblasts, chondrocytes, vascular smooth muscle cells, pneumocytes, kidney cells, and fibroblasts.3-8 Although several noncalcified tissues express MGP mRNA at a higher level than bone, significant levels of the protein have been found only in bone and calcified cartilage.<sup>4,9</sup> This observation suggests that the protein may accumulate at sites of calcification and that much of the protein secreted by noncalcified tissues probably escapes to plasma, where MGP is found at concentrations of 0.3 to 1  $\mu$ g/mL depending on the species. MGP is the target of several additional posttranslational modifications in addition to  $\gamma$ -carboxylation. Specific proteolytic cleavage at a conserved dibasic site in the C-terminal region has been observed in MGP isolated from human, bovine, and shark tissues,<sup>10</sup> and conserved phosphorylation of 3 phosphoserine residues in the N-terminal region has been found in MGP from shark, rat, cow, and human tissues.<sup>11</sup>

The function of MGP was recently explored by targeted gene deletion in the mouse.<sup>12</sup> Investigators found that MGP-deficient mice are normal at birth but that calcification of the arterial media first appears at 1 week of age and progresses rapidly to encompass the entire media by 3 weeks of age. The extensively

calcified arteries are fragile, and most of the MGP-deficient mice die of exsanguination by 3 to 6 weeks of age. Surviving animals begin to show signs of bone involvement, including calcification of growth plate cartilage with growth plate closure, osteopenia, and bone fracture, after 3 weeks of age.

The objective of the current study was to determine the effect of the vitamin K antagonist warfarin on the calcification of soft tissues in the rat. The procedures used have their genesis in earlier studies with the vitamin K-dependent bone Gla protein (osteocalcin),<sup>13,14</sup> which led to the discovery that vitamin K cannot counteract the effect of warfarin in extrahepatic tissues.<sup>15</sup> This result is in marked contrast to the well-established ability of vitamin K to counteract the effect of warfarin on the synthesis of blood coagulation factors by the liver.<sup>16</sup> The fundamental dichotomy in the ability of vitamin K to counteract the effect of warfarin in different tissues allows continuous maintenance of animals on doses of warfarin sufficient to severely inhibit  $\gamma$ -carboxylation of proteins synthesized by extrahepatic tissues while maintaining normal blood coagulation times.14,17 We report that a modified version of the warfarin plus vitamin K protocol used in the original studies with bone Gla protein produces a rapid, extensive calcification of elastic lamellae in the aorta and other arteries that is quite similar to the phenotype of the MGP-deficient mouse.

## Methods

### Materials

Vitamin  $K_1$  (phylloquinone) and warfarin were purchased from Sigma Chemical Co. For injections, stock solutions of vitamin  $K_1$  (10

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mg/mL) were prepared and stored in sterile, foil-wrapped containers at 4°C. Stock solutions of sodium warfarin (50 mg/mL) were prepared and stored at 4°C. Simonsen albino rats (derived from Sprague-Dawley rats) were purchased from Simonsen Laboratories (Gilroy, Calif).

#### Methods

Calcified tissues were dried, weighed, and extracted with a 10-fold excess (wt/vol) of 10% formic acid for 16 hours at 4°C. MGP levels in the acid extracts and serum were determined by radioimmunoassay as described previously.<sup>18</sup> Calcium levels in acid tissue extracts were determined colorimetrically by using cresolphthalein complex 1 (Sigma), and phosphate levels were determined colorimetrically as described previously.<sup>19</sup> Tissue sectioning and staining were performed by Biological Testing Service. For radiographs, the carotid arteries and abdominal aorta were removed at necropsy, cleaned of nonvascular tissue, and photographed with a Hewlett-Packard model 4380N Flexitron x-ray system. Prothrombin times were determined by using the thromboplastin time with calcium reagents and following the manufacturer's procedure (Sigma).

For Northern blot analysis, total RNA was isolated from thoracic aortas and normal rat kidney cells<sup>3</sup> with an RNA STAT-60 kit (Tel-Test B).Forty micrograms of total RNA from each sample was fractionated on a 1% formaldehyde-agarose gel in MOPS buffer and transferred onto a Hybond-N membrane (Amersham). After 3 hours of prehybridization in 50% formamide,  $5 \times SSC$ ,  $5 \times$  Denhardt's solution, and 100  $\mu$ g/mL denatured salmon sperm DNA at 42°C, the blot was hybridized with a randomly primed, <sup>32</sup>P-labeled cDNA probe for rat MGP<sup>20</sup> for 16 hours. The membrane was washed 3 times for 1 hour each with 0.1× SSC containing 0.1% SDS at 65°C, and then exposed to x-ray film.

#### **Maintenance of Animals**

Study procedures were reviewed and approved by the University of California, San Diego, Animal Subjects Committee before initiation. Male Sprague-Dawley rats were given free access to rodent diet 5001 (Purina Mills), which is 0.67% phosphorus and 0.95% calcium by weight. This diet contains 500 µg phylloquinone/kg and has no added menadione. All injections were administered subcutaneously in the back of animals essentially as described previously.<sup>13-13</sup> Twenty-four and 48 hours before the first warfarin injection, all rats received doses of 1.5 mg vitamin K<sub>1</sub>/100 g body weight. Previous studies have shown that this loading dose of vitamin K is necessary to prevent bleeding during the first week of warfarin treatment.<sup>15</sup> The first warfarin dose, 15 mg/100 g, and 1.5 mg vitamin K<sub>1</sub>/100 g were administered at 8 AM when rats reached 42 days of age. A second 15 mg/100 g dose of warfarin was administered at 8 PM with no accompanying vitamin K. This routine was maintained every day until termination of the experiment. To reduce trauma at injection sites, 25-gauge needles were used for all injections, and subcutaneous injection sites were rotated between the 4 quadrants of the back. Animals were killed by exsanguination while they were under metofane anesthetic, and selected tissues were removed and fixed in 10% buffered formalin or frozen at -70°C for later studies.

The first 5-week study was performed with 10 rats in the warfarin treatment group and 10 rats in the control group; rats in the control group received daily injections of the same dose of vitamin  $K_1$  but no warfarin. In a subsequent study, 2 rats were maintained on warfarin for 1 week and 2 for 2 weeks. In a third study, 2 rats were maintained on warfarin for 3 weeks, 2 for 4 weeks, and 2 for 5 weeks. Finally, in the course of developing the dose regimen for the revised warfarin protocol, we maintained 4 animals on the standard dosage of 15 mg warfarin/100 g every 12 hours for 3 weeks with either the standard 1.5 mg vitamin  $K_1/100$  g per day or one half of this dose.

### Results

**Modification of Warfarin Plus Vitamin K Protocol** In the initial study of the effect of experimentally induced vitamin K deficiency on arterial calcification, 42-day-old male



**Figure 1.** Effect of warfarin treatment on growth. Six-week-old male Sprague-Dawley rats were treated with injections of vitamin K alone every 24 hours or vitamin K every 24 hours and warfarin every 12 hours. Data are average weights determined for rats in each group. (See Methods for details.)

rats were treated for 6 weeks with the daily dosages of warfarin and vitamin K, which, in previous studies,<sup>14</sup> lowered levels of bone Gla protein in bone to 2% of normal levels. The presence of mineralization in the arteries of warfarin-treated rats was not detected, either as an increase in calcium levels of the aorta or as the presence of von Kossa staining in histological sections of the aorta, femoral artery, or carotid artery (data not shown). One possible explanation for the failure to find an increase in arterial calcification in these warfarin-treated rats is that the protocol developed to reduce levels of bone Gla protein in bone to 2% of normal levels did not produce a comparably significant defect in  $\gamma$ -carboxylation of MGP. Another possibility is that even 2% of normal MGP  $\gamma$ -carboxylation may be sufficient to prevent significant calcification in the arteries of warfarin-treated rats. In either case, the solution to the arterial calcification problem would be to further reduce the level of MGP  $\gamma$ -carboxylation. We therefore increased the warfarin dose by a factor of 2 and administered this dose every 12 hours rather than every 24 hours. The total daily dose of warfarin was thereby increased 4-fold.

Rats maintained for 5 weeks on this revised warfarin plus vitamin K protocol were quite healthy. As shown in Figure 1, the modified warfarin treatment protocol had no effect on the rate at which the initially 6-week-old rats gained weight over the 5 weeks of the experiment. There was also no effect of 5 weeks of warfarin treatment on tibial length, which was 3.9 cm for both the warfarin-treated and control rats, and there was no radiographic evidence of growth plate fusion (radiographs not shown). The absence of an effect of warfarin on bone growth is in agreement with results of earlier studies, which showed that warfarin causes growth plate fusion only in rats 4 months of age or older.<sup>21</sup> Serum chemistry tests, performed after 4 weeks of treatment, did not detect a significant difference between the warfarin-treated and control groups in any of the measured parameters, including serum calcium and phosphorus levels. As in earlier experiments,14,15 concurrent treatment of animals with high doses of vitamin K was able to counteract the effect of warfarin on the carboxylation of blood coagulation factors by the liver, as shown by the fact that prothrombin times and hematocrits remained normal throughout the experiment and that there was no single instance of uncontrolled bleeding at a site of injection.



**Figure 2.** Effect of warfarin treatment on calcification of the aorta. Sections show the abdominal aorta of rats treated with warfarin every 12 hours and vitamin K every 24 hours for 2, 3, 4, and 5 weeks. The segment of the abdominal aorta between the renal branch and the iliac bifurcation was removed at necropsy and fixed in 10% buffered formalin, and longitudinal sections were stained with von Kossa stain to detect areas of mineralization.

## Effect of Warfarin on Calcification of Arteries

The time course of arterial calcification was investigated in 42-day-old male rats. The 10 experimental animals were given warfarin every 12 hours and vitamin K every 24 hours; the 10 control rats were given vitamin K alone every 24 hours. Two animals were killed from each group every week, and the aorta, heart, lungs, kidneys, and carotid and femoral arteries were fixed in formalin. No calcification was detected by von Kossa staining of abdominal aorta sections after 1 week of warfarin treatment. By 2 weeks of treatment, however, longitudinal sections of the abdominal aorta contained darkly stained regions of calcification that were confined to linear structures within the aortic media (Figure 2). These linear structures were identified as the elastic lamellae of the aortic media by staining serial sections of the abdominal aorta for elastin and mineral. By 3 weeks of treatment, the number of calcified regions in the abdominal aorta had increased significantly (Figure 2). Each calcified region was focal, with areas of intense von Kossa staining for mineral interspersed with regions of the aortic media apparently free of any calcification. By 4 and 5 weeks of treatment, the intensity of von Kossa staining in regions of calcification increased further, and some calcified regions eventually became so hard that there was fragmentation during sectioning (Figure 2). A similar time course and pattern of medial calcification were also observed in von Kossa-stained sections of the coronary, carotid, and femoral arteries of warfarin-treated rats (micrographs not shown).

We found that the time course of warfarin-induced arterial calcification is remarkably reproducible. In subsequent experiments, we examined the extent of arterial calcification in rats maintained on warfarin for 1, 2, 3, 4, or 5 weeks, and the results of these experiments, performed over a 4-month period with 2 rats per time point, were indistinguishable from the time course depicted in Figure 2. In a separate experiment, we investigated the effect of reducing the maintenance dose of vitamin K by a factor of 2 on the extent of calcification at 3 weeks of warfarin treatment. Four animals were maintained on warfarin, 2 with the standard dose of vitamin K and 2 with

half of this dose. Calcification of the abdominal aorta in the 2 groups was indistinguishable, as might have been anticipated from the previously documented inability of vitamin K to counteract the effect of warfarin in extrahepatic tissues.<sup>15</sup> Because prothrombin times were prolonged 1.2-fold to 1.6-fold in the group receiving half the standard dose of vitamin K, we used the standard dose of 1.5 mg vitamin K/100 g body weight in all other experiments reported here.

An overview of the pattern of arterial calcification in the warfarin-treated rat can be obtained either by visual inspection of the tissues with a dissecting microscope or by examination of radiographs of the arteries. Visual detection of calcification is easiest in the carotid artery, which normally has a translucent aspect. As shown in Figure 3, calcification of the carotid artery in warfarin-treated rats appears as discontinuous, whitish deposits against a background of otherwise translucent tissue. These white regions often form irregular bands around the artery that appear to be hard when the tissue is manipulated. After the carotid artery shown in



**Figure 3.** Photograph of the carotid artery (in situ) after 4 weeks of warfarin treatment (taken through a dissecting microscope). Note the white bands of circumferential calcification. (The lines near the artery are coat hairs from the initial dissection of the animal.)



## **Carotid Arteries**



# **Abdominal Aorta**

**Figure 4.** Radiographs of the aorta and carotid arteries from a rat treated with warfarin for 5 weeks. The abdominal aorta and both carotid arteries were removed at necropsy and radiographed. Note the punctate areas of mineralization in each artery.

Figure 3 was fixed and sectioned longitudinally, calcification appeared as focal deposits similar to those shown in Figure 2. We therefore conclude that focal calcification seen in longitudinal sections can, in some instances, represent crosssectional cuts through circumferential bands of calcification. Calcification can easily be detected radiographically in the aorta and carotid artery by 5 weeks of warfarin treatment (Figure 4). In each instance, the calcification seen in the radiograph is markedly discontinuous, with areas of calcification separated by apparently uncalcified regions of the artery. Radiographs of the carotid artery and aorta also show that calcification often appears as irregular bands rather than as punctate foci.

Chemical analyses were performed on the aorta and selected additional tissues from rats maintained for 4 weeks on the revised warfarin plus vitamin K protocol. As shown in

#### TABLE 1. Effect of Modified Warfarin Treatment Protocol on Level of Calcium and MGP in Rat Tissues

Tissue	Calcium, μg/mg Tissue	MGP, ng/mg Tissue	
Aorta			
Control	0.40	8	
Warfarin	1.53	105	
Metaphysis			
Control	215	107	
Warfarin	261	21	
Kidney			
Control	0.20	ND	
Warfarin	0.75	ND	

ND indicates not determined. Rats were treated for 4 weeks with warfarin plus vitamin K or vitamin K alone. At necropsy, the thoracic aorta, kidney, lung, and metaphysis and epiphysis of the proximal tibia were removed from each rat, dried, and weighed. Each tissue was demineralized with a 10-fold excess (wt/vol) of 10% formic acid, and the acid extracts were analyzed for calcium and MGP (see Methods). Results are average values for 2 animals from each group.

Table 1, warfarin treatment increased the total level of calcium in acid extracts of the aorta by  $\approx$ 4-fold. Phosphate levels in the acid extracts were increased to a similar extent (data not shown). Warfarin treatment also caused a 4-fold increase in the level of calcium in the kidney (Table 1).

## Effect of Warfarin on MGP Expression

The effect of warfarin treatment on MGP expression in arteries was evaluated in 2 ways. Northern blot analysis of MGP mRNA levels was performed on RNA isolated from the aortas of rats treated for 4 weeks with warfarin and vitamin K or vitamin K alone. As shown in Figure 5, warfarin treatment significantly increased the level of MGP mRNA expression in the aorta of warfarin-treated rats. MGP antigen levels were also determined by radioimmunoassay of the acid demineralization extracts of the aorta of rats maintained for 4 weeks



**Figure 5.** Northern blot analysis of MGP mRNA levels in the aorta of rats treated for 4 weeks with warfarin and vitamin K or vitamin K alone. RNA was extracted from the thoracic aorta, and 40  $\mu$ g of total RNA from each aorta was run on a 1.4% formaldehyde-agarose gel, blotted onto a Nytran membrane, and hybridized with a <sup>32</sup>P-labeled MGP cDNA and a <sup>32</sup>P-labeled cDNA for GAPDH. Lane 1, Total RNA from normal rat kidney cells, an abundant source of MGP mRNA.<sup>3,46</sup> Lanes 2 to 5, Total RNA from the thoracic aorta of 2 warfarin-treated and 2 control rats.

TABLE 2. Effect of Modified Warfarin Treatment Protocol on Level of MGP in Serum

Treatment Group	Serum MGP Level, ng/mL
Warfarin+vitamin K (n=24)	119±30
Vitamin K alone (n=9)	347±44

Values are mean $\pm$ SD. The level of MGP was determined by radioimmunoassay of serum from rats treated for 1 to 5 weeks with warfarin plus vitamin K or vitamin K alone.

on the warfarin and control protocols. As shown in Table 1, warfarin treatment increased the level of MGP in the aorta of the warfarin-treated animals by 13-fold.

The dramatic accumulation of MGP antigen in the calcified aortas of the warfarin-treated rats suggests that  $\gamma$ -carboxylation of the protein may not be essential for its accumulation in calcified tissue. To test this hypothesis further, we examined the level of MGP in the segment of the metaphysis that had formed in the course of bone elongation during the 4 weeks of warfarin treatment. As shown in Table 1, MGP antigen accumulated in the regions of bone calcification formed during the interval of warfarin treatment, but the level of MGP per unit of mineral calcium was reduced by  $\approx$ 5-fold compared with the level in control bone tissue. A similar reduction in MGP levels was also seen in SDS gels of acid-extracted proteins from this region of the metaphysis of rats treated with warfarin for 4 weeks (data not shown).

Because warfarin-induced calcification increases the level of MGP mRNA and antigen in vascular tissues, it might be anticipated that there would also be an increase in the level of MGP antigen in blood. As shown in Table 2, however, the level of serum MGP was actually almost 3-fold lower in warfarin-treated rats. This decrease is serum MGP did not change during the 5 weeks of warfarin treatment and was therefore unrelated to arterial calcification. It seems more likely that the decrease in serum MGP levels was due either to increased clearance of the noncarboxylated protein or to a warfarin-induced decrease in the secretion of MGP from cells similar to the warfarin-induced decrease in the secretion of prothrombin reported in rat hepatocytes.<sup>22,23</sup>

## Effect of Warfarin on Calcification of Heart, Lung, and Kidney

Because the highest levels of MGP mRNA have been found in the heart, lung, and kidney of rats of this age,<sup>3</sup> it seems logical to conclude that each of these tissues may, under some circumstances, be prone to calcify. To evaluate the effect of warfarin treatment on the calcification of these tissues, the heart, lungs, and kidneys were removed at necropsy from rats treated for 4 weeks with warfarin and vitamin K or vitamin K alone. Sections of each tissue were then subjected to von Kossa staining to detect areas of mineralization. In kidney, calcification was found in the elastic lamellae of arteries in the renal hilum but not in any other structure (not shown). In heart, calcification was found only in the elastic lamellae of the coronary artery (not shown) and aortic heart valve (Figure 6). In lung, calcification was found in the elastic lamellae of a number of arteries but not in any other structures. Although we did not evaluate, by histological methods, the calcification



**Figure 6.** Effect of warfarin treatment on calcification of the aortic heart valve. Hearts were removed at necropsy from rats treated for 4 weeks with warfarin and vitamin K or vitamin K alone. Sections were then cut throughout each heart and stained with von Kossa stain to detect areas of mineralization. The only regions of calcification were found in the aortic heart valve (this figure) and the coronary arteries (not shown) of warfarin-treated rats.

of any other soft tissues in warfarin-treated rats, it should be noted that such calcifications, if present, were not sufficiently extensive to be detected in whole-animal radiographs of rats treated with warfarin for 5 weeks.

#### Discussion

### Comparison of the Effect of Warfarin in Rats With the Phenotype of the MGP-Deficient Mouse

As noted above, targeted deletion of the MGP gene in mice causes calcification of the major arteries that can first be detected at 1 week of age.<sup>12</sup> This calcification begins within the elastic lamellae of the arterial media and progresses to the point that the arteries become rigid tubes that are fragile and rupture, causing death by exsanguination in most MGP-deficient mice by 3 to 6 weeks of age. Tracheal ring cartilage also calcifies in MGP-deficient mice by 2 to 3 weeks of age. After 3 weeks of age, surviving mice grow more slowly, partly because of the abnormal calcification of growth plate cartilage, and eventually have osteopenia and bone fractures.<sup>12</sup>

Most of the salient phenotypic features that appear early in MGP-deficient mice are also found to a somewhat muted degree in warfarin-treated rats. The same arteries also calcify in warfarin-treated rats, and calcification also begins within the elastic lamella of the aortic media. Calcification progresses more slowly in warfarin-treated rats, however, and is focal and discontinuous in appearance, in contrast to the calcification seen in MGP-deficient mice, which forms a continuous calcification sheet that encompasses the entire arterial media. We believe that the muted extent of the calcification induced by warfarin in rats is due to the fact that  $\gamma$ -carboxylation of MGP was not completely inhibited by the warfarin doses we used. It is also possible, however, that the protein is in fact completely nony-carboxylated at these warfarin doses and that the non- $\gamma$ -carboxylated protein retains some residual activity as a calcification inhibitor. The major difference between the phenotype of the MGP-deficient mouse and the warfarin-treated rat is the absence of the reduced bone growth, osteopenia, and fractures in the warfarin-treated rat that are features of the mouse phenotype after 3 weeks of age. One possible explanation for this difference is that the defect in MGP  $\gamma$ -carboxylation produced by warfarin has no effect on bone metabolism. It is also possible that rats the age of those used in the current study are more similar to mice in the first 3 weeks of life, a period in which bone growth abnormalities were not observed in the MGP-deficient mouse.<sup>12</sup> This possibility is supported by results of earlier studies of rats maintained on warfarin, which found that warfarin treatment causes abnormal calcification of growth plate cartilage and attendant cessation of longitudinal bone growth that are essentially identical to those seen in MGP-deficient mice but that occur only in rats 4 months of age or older.<sup>14,21</sup>

A puzzling feature of both the MGP-deficient mouse phenotype and the warfarin-treated rat syndrome is the absence of evidence of calcification at other tissue sites known to express MGP at a high rate. One possibility is that rodents maintained on carefully designed diets in the absence of stresses they would be expected to encounter in nature may have very little tendency to calcify most soft tissues. One test of this hypothesis will be to examine the calcification of selected soft tissues in warfarin-treated animals subjected to dietary manipulations, such as altered levels of calcium and phosphate. In this context, it is worth noting that kidney calcification has long been a problem with rodent chows and that the elimination of this problem in diets such as the one used here has required careful adjustment of the calcium and phosphate contents of the diet.<sup>24-26</sup> It is therefore possible that diets with different calcium and phosphate contents, and different ratios of calcium to phosphate, will lead to accelerated calcification of kidneys and other soft tissues in the warfarin-treated rat. Another test of the hypothesis that MGP functions as a calcification inhibitor in tissues other than arteries will be to subject warfarin-treated rats to stresses known to cause systemic or focal calcification at nonvascular sites in humans, such as uremia, tissue trauma, and cancer.

## Arterial Calcification and the Mechanism of MGP Action

Previous studies with MGP-deficient mice and the current study with warfarin-treated rats provide strong evidence that MGP functions in vivo as a calcification inhibitor. To our knowledge, no other protein has been established to have this function in vivo, and no other protein has a targeted deletion that causes increased calcification of any tissue. The fact that calcification of arteries is so extensive in animals that either do not express this gene or cannot  $\gamma$ -carboxylate this protein argues strongly against the hypothesis that there is an equivalently active inhibitor of calcification in vascular tissue and that MGP has a mere backup function in preventing calcification of this tissue. It seems more likely that MGP is central to the process by which the calcification of arteries is normally inhibited in vivo.

The origin of calcification within the elastic lamellae of the media of arteries in MGP-deficient mice and warfarin-treated rats suggests that elastic lamellae of arteries are prone to calcify in vivo. This hypothesis is supported by studies showing that the elastic lamellae are the first sites of arterial calcification in humans and that the accumulation of calcification within these elastic lamellae begins by the second decade of life and increases progressively with age.<sup>27-32</sup> Elastin has also been shown to be a potent and reproducible initiator of calcification in vitro.<sup>33-39</sup> In vitro calcification of elastin occurs in serum and neutral buffered solutions at physiological concentrations of calcium and phosphate. This finding is in marked contrast to results of most studies on macromolecule- or cell culture– induced calcification, which relied on supraphysiological concentrations of calcium and phosphate to achieve calcification.

The focal nature of calcification in the aorta of warfarintreated rats may provide insight into the mechanism by which MGP normally inhibits arterial calcification in vivo. Focal mineralization is not observed in MGP-deficient mice, presumably because there is so little ability to inhibit calcification that all of the many crystal nuclei generated by elastin can grow and thereby form a solid sheet of medial calcification. In warfarin-treated rats, the ability to inhibit calcification would presumably remain sufficient enough to inactivate most crystal nuclei, probably by a process that involves direct MGP binding to the mineral surface. A few nuclei would, however, grow and attain the critical calcification size at which the generation of seed crystals exceeds the ability of the residual MGP activity to inhibit the calcification chain reaction, and calcification would spread focally within and eventually between the elastic lamellae at this site.

The detailed MGP mechanism that best fits the available data is one in which the protein binds tightly and selectively to hydroxyapatite crystal nuclei and prevents their growth and ability to seed daughter crystals. It is also possible that the putative MGP-coated crystals may be recognized by cells in the vicinity of elastin nucleation sites and that cells may clear some of these coated crystals and dissolve them within the cell. The calcification-inhibitory activity of MGP is likely to be highly regulated, which may explain the carboxyl-terminal processing of MGP<sup>10</sup> and the presence of phosphoserine at 3 conserved sites of partial phosphorylation.<sup>11</sup> This inhibitory activity must be driven by an unusually strong association of the protein with the mineral nuclei. We speculate that this association includes strong lateral interactions between adjacent, mineral-bound MGP molecules and that this lateral interaction reflects the ability of MGP to self-associate. We previously showed that MGP is highly insoluble in vitro because of its tendency to self-associate and that this property of the protein is conserved in MGP from species ranging from shark to human.1,9

Effect of Warfarin on MGP Expression in Arteries The current study demonstrated that warfarin causes an increased level of MGP mRNA expression and accumulation of MGP antigen in aorta. The most likely explanation for the increase in MGP mRNA in the aorta is increased synthesis of the protein by vascular smooth muscle cells in response to calcification of the aortic media. Vascular smooth muscle cells are known to express MGP mRNA in cell culture<sup>6</sup> and at sites of calcification in atherosclerotic plaques.<sup>40,41</sup> In the anatomy of the rat aortic media, vascular smooth muscle cells are interspersed between each elastic lamella. Because calcification begins within the elastic lamella, it is possible that vascular smooth muscle cells sense the presence of nearby regions of calcifying elastin and respond with increased MGP expression to help arrest further growth of the calcification. Studies are needed to correlate cellular levels of MGP mRNA in smooth muscle cells of the artery with the proximity of these cells to actively calcifying elastic lamellae and to identify the mechanisms by which cells sense nearby sites of ongoing calcium accretion.

The dramatic 13-fold increase in the level of MGP antigen in aorta after 4 weeks of warfarin treatment probably arose from MGP secreted by vascular smooth muscle cells in the aorta, and MGP was probably bound to the calcification itself. This latter hypothesis is supported by the fact that MGP can be released from the aorta only by demineralization of the tissue. If MGP in fact accumulates on the surface of crystals during the course of arterial calcification, it may be more insightful to express MGP content of the aorta per unit of mineral calcium rather than per milligram dry weight. When expressed this way, the MGP content of calcifying aorta is >20-fold higher than that of bone (Table 1) or any other tissue examined to date.<sup>4</sup> The simplest explanation for this observation is that the local concentration of MGP may be maintained at a far higher level near sites of arterial mineralization than is the case in bone.

The fact that MGP accumulates in the bone metaphysis and aorta of the warfarin-treated rat (Table 1) despite the probable under– $\gamma$ -carboxylation of the protein indicates that  $\gamma$ -carboxylation of MGP is not required for binding to calcifying tissues and possibly to the apatite in these tissues. In support of this hypothesis, we found that thermal decarboxylation of MGP in vitro does not affect the ability of the protein to bind to hydroxyapatite from serum. If non– $\gamma$ -carboxylated MGP does accumulate in the calcifying aorta because of its ability to bind to hydroxyapatite in this tissue, it is unclear why the non– $\gamma$ -carboxylated protein fails to arrest the growth of mineral.

#### Warfarin Dose and Artery Calcification

The dramatic difference between the complete absence of artery calcification with a warfarin dose given once every 24 hours and the extensive and rapid calcification of arteries with twice this dose administered every 12 hours may be due in part to the rapid clearance of warfarin from plasma in the rat. Previous studies have shown that warfarin is cleared from the plasma of rats with a half-time of 5 hours and that the half-time for warfarin clearance is not affected by the age or sex of the animal.<sup>42</sup> The half-time for warfarin clearance is also not affected by warfarin dose,<sup>43</sup> and the R and S enantiomers of warfarin are cleared at about the same rate.44 Because of the rapid clearance of warfarin from plasma, the concentration of warfarin achieved shortly after a single subcutaneous injection of the drug will fall to 20% of this value at 12 hours and to <4% of this value by 24 hours. A daily injection schedule, as used in our earlier version of the warfarin plus vitamin K protocol,<sup>13–15,21</sup> will therefore produce marked oscillations in the plasma concentration of the drug. If the effect of warfarin on  $\gamma$ -carboxylation of proteins in extrahepatic tissues is proportional to the plasma concentration of the drug, the daily injection protocol will produce severe impairment of  $\gamma$ -carboxylation shortly after warfarin administration, followed by substantial recovery by the time of the next injection at 24 hours. The 12-hour injection protocol used here would, in contrast, cause a 12-hour oscillation between the warfarin concentration shortly after injection and a concentration that is 20% of this level and therefore would allow substantially less time at the lower plasma warfarin levels for recovery of extrahepatic protein  $\gamma$ -carboxylation status.

An argument can be made that continuous growth of calcification nuclei into large mineral deposits is in fact more dependent on the minimum level of warfarin in plasma during each 24-hour period than on the maximum level. This is because the process of mineralization is an autocatalytic process, much like a chain reaction, which needs a continuous rate of crystal nuclei generation to sustain growth. When the level of MGP  $\gamma$ -carboxylation rises to the point that the activity of MGP secreted near the site of calcification is sufficient to reduce the level of crystal nuclei generation below that needed to sustain the mineralization chain reaction, the growth of the mineral phase will cease. Because normally y-carboxylated MGP would now presumably coat and thereby inactivate mineral surfaces, a subsequent warfarin dose would be unable to restart mineral growth at this site. The daily dose of warfarin required to cause arterial calcification would therefore be determined by the need to keep the plasma concentration of drug above a given value throughout each 24-hour period.

## Vitamin K Dose, Blood Coagulation, and Artery Calcification

The change from a given warfarin dose administered every 24 hours to twice this dose given every 12 hours affects the dose of vitamin K needed to counteract the effect of warfarin on blood coagulation. When animals were maintained on the new, higher warfarin dose regimen and the former vitamin K dose of 0.75 mg/100 g, blood coagulation times were longer than normal and there were occasional incidents of bleeding around subcutaneous injection sites. Doubling this vitamin K dose restored normal coagulation times and eliminated bleeding at injection sites. The ability of vitamin K to counteract the effect of warfarin on the  $\gamma$ -carboxylation of blood coagulation factors by the liver was established in earlier studies.<sup>15,16</sup>

In contrast, doubling the vitamin K dose did not significantly affect the extent of artery calcification produced by the new, higher warfarin dose regimen. The inability of vitamin K to counteract the effect of warfarin on the synthesis of bone Gla protein by osteoblasts in bone was noted in previous studies,<sup>15</sup> and it has been speculated that vitamin K can counteract the effect of warfarin only at sites of blood coagulation factor synthesis in hepatocytes.<sup>15</sup> The results of the current study support this hypothesis and indicate that arteries and bone cells share an epoxide reductase recycling mechanism that is inhibited by warfarin in a way that cannot be affected by even high doses of vitamin K.

### Does the Defective Carboxylation of MGP Accelerate Soft Tissue Calcification in Humans?

Results of the current study indicate that it is possible that a defect in the  $\gamma$ -carboxylation of MGP in humans could accelerate calcification of arteries and heart valves. The possible importance of vitamin K intake to arterial calcification is supported by a recent report that lower dietary intake of vitamin K is correlated with increased aortic calcification in humans.<sup>45</sup> To further test the hypothesis that defective  $\gamma$ -carboxylation of MGP contributes to arterial calcification and to establish the vitamin K intake required to repair this defect, we are currently developing assays to measure the  $\gamma$ -carboxylation status of plasma MGP analogous to the hydroxyapatite-binding assay we developed earlier for determining the  $\gamma$ -carboxylation status of bone Gla protein in serum.<sup>13</sup>

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