

Warfarin-Induced Artery Calcification Is Accelerated by Growth and Vitamin D

Paul A. Price, Samuel A. Faus, Matthew K. Williamson

Abstract—The present studies demonstrate that growth and vitamin D treatment enhance the extent of artery calcification in rats given sufficient doses of Warfarin to inhibit γ -carboxylation of matrix Gla protein, a calcification inhibitor known to be expressed by smooth muscle cells and macrophages in the artery wall. The first series of experiments examined the influence of age and growth status on artery calcification in Warfarin-treated rats. Treatment for 2 weeks with Warfarin caused massive focal calcification of the artery media in 20-day-old rats and less extensive focal calcification in 42-day-old rats. In contrast, no artery calcification could be detected in 10-month-old adult rats even after 4 weeks of Warfarin treatment. To directly examine the importance of growth to Warfarin-induced artery calcification in animals of the same age, 20-day-old rats were fed for 2 weeks either an ad libitum diet or a 6-g/d restricted diet that maintains weight but prevents growth. Concurrent treatment of both dietary groups with Warfarin produced massive focal calcification of the artery media in the ad libitum-fed rats but no detectable artery calcification in the restricted-diet, growth-inhibited group. Although the explanation for the association between artery calcification and growth status cannot be determined from the present study, there was a relationship between higher serum phosphate and susceptibility to artery calcification, with 30% higher levels of serum phosphate in young, ad libitum-fed rats compared with either of the groups that was resistant to Warfarin-induced artery calcification, ie, the 10-month-old rats and the restricted-diet, growth-inhibited young rats. This observation suggests that increased susceptibility to Warfarin-induced artery calcification could be related to higher serum phosphate levels. The second set of experiments examined the possible synergy between vitamin D and Warfarin in artery calcification. High doses of vitamin D are known to cause calcification of the artery media in as little as 3 to 4 days. High doses of the vitamin K antagonist Warfarin are also known to cause calcification of the artery media, but at treatment times of 2 weeks or longer yet not at 1 week. In the current study, we investigated the synergy between these 2 treatments and found that concurrent Warfarin administration dramatically increased the extent of calcification in the media of vitamin D-treated rats at 3 and 4 days. There was a close parallel between the effect of vitamin D dose on artery calcification and the effect of vitamin D dose on the elevation of serum calcium, which suggests that vitamin D may induce artery calcification through its effect on serum calcium. Because Warfarin treatment had no effect on the elevation in serum calcium produced by vitamin D, the synergy between Warfarin and vitamin D is probably best explained by the hypothesis that Warfarin inhibits the activity of matrix Gla protein as a calcification inhibitor. High levels of matrix Gla protein are found at sites of artery calcification in rats treated with vitamin D plus Warfarin, and chemical analysis showed that the protein that accumulated was indeed not γ -carboxylated. These observations indicate that although the γ -carboxyglutamate residues of matrix Gla protein are apparently required for its function as a calcification inhibitor, they are not required for its accumulation at calcification sites. (*Arterioscler Thromb Vasc Biol.* 2000;20:317-327.)

Key Words: Warfarin ■ vitamin K ■ vitamin D ■ artery calcification ■ matrix Gla protein

The current studies were performed to identify those factors that influence the extent of artery calcification in rats treated with Warfarin, a vitamin K antagonist that inhibits the formation of the calcium-binding amino acid, γ -carboxyglutamic acid (Gla), in specific proteins. The target for Warfarin treatment in these investigations is matrix Gla protein (MGP), a vitamin K-dependent protein that inhibits artery calcification and that is secreted by vascular smooth muscle cells and macrophages in the artery.

MGP is a 10-kDa secreted protein that contains 5 residues of γ -carboxyglutamic acid.^{1,2} MGP was originally discovered in demineralization extracts of bone, but it 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,^{3,4} 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 do ex-

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press MGP mRNA at a higher level than bone, significant levels of the protein itself have only been found in bone and calcified cartilage.^{4,9} 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 0.3 to 1 $\mu\text{g/mL}$, depending on the species. MGP is the target of several other posttranslational modifications in addition to γ -carboxylation. Specific proteolytic cleavage at a conserved dibasic site in the C-terminal region has been observed for MGP isolated from human, bovine, and shark tissues,^{9,10} and conserved phosphorylation of 3 phosphoserine residues in the N-terminal region has been found in MGP from shark, rat, cow, and human tissues.¹¹

Recent genetic and biochemical studies have established MGP as the first protein known to act as a calcification inhibitor *in vivo*. In humans, defects in the MGP gene that predict a nonfunctional MGP protein have been shown to be responsible for Keutel syndrome.¹² This syndrome is a rare, inherited disease characterized by abnormal calcification of cartilages, including costal, nasal, auricle, tracheal, and growth plate cartilage; by nasal hypoplasia and brachytelephalangia; and by multiple peripheral pulmonary artery stenoses.^{13,14} In mice, targeted deletion of the *MGP* gene causes rapid calcification of the elastic lamellae of the arterial media that begins at birth and is sufficiently extensive by 3 to 6 weeks of age that the arteries become rigid tubes that fracture, causing death by exsanguination in most of the affected mice by 6 weeks of age.¹⁵ MGP-deficient mice also display abnormal calcification of growth plate and tracheal cartilage. Finally, treatment of rats with the vitamin K antagonist Warfarin at doses that inhibit the γ -carboxylation of MGP causes rapid calcification of elastic lamellae of arteries and of aortic heart valves and increased expression of MGP mRNA in the calcifying artery.¹⁶

Calcification is a common finding in the pathophysiology of the aging human artery and heart valve and is associated with several cardiovascular disease states. Calcification is almost universally associated with atherosclerotic plaques, and calcification at plaque sites is typically so extensive as to be rigid and bonelike in consistency.^{17–19} Calcification also occurs in human aortic heart valves^{20–23} and is usually seen in valves removed in the course of valve replacement surgery. The human cardiovascular system is the site of 2 additional kinds of calcification. In some individuals, arteries become rigidly calcified in a linear fashion, to an extent such that the artery resembles a rigid tube. This calcification, which has been termed Monckeberg's syndrome, is confined to the media of the artery and is not necessarily associated with atherosclerotic plaque formation.^{24,25} In all individuals, there is a less obvious, diffuse calcification of the artery, a calcification that is confined to the artery media and that does not result in artery rigidity.^{26–31} Such diffuse calcifications of the artery can be detected as early as the second decade of life and accumulate with age, becoming $\approx 15\%$ of the dry weight of the media by the eighth decade of life.³¹ We have analyzed the MGP levels in all of these cardiovascular calcifications, and in each instance we have found MGP at levels higher than those found in any normal human tissue, including bone (personal observation, manuscript in preparation).

A major objective of our research is to understand the role of MGP in human cardiovascular calcifications. The current investigations are 1 of several designed to identify the factors that act synergistically with Warfarin to accelerate artery calcification in the rat, with the goals of further understanding the role of MGP in this process and of identifying the physiological circumstances in which it might, in future studies, be appropriate to search for the possible association between Warfarin treatment and increased risk of cardiovascular calcification in humans. In the first series of experiments presented here, we have determined the effect of age and growth rate on artery calcification in the Warfarin-treated rat. These studies reveal that Warfarin-induced artery calcification occurred only in the growing animal and was not seen either in older, nongrowing rats or in young rats whose growth was temporarily arrested by a calorically restricted diet. In the second set of experiments, we examined the effect of concurrent Warfarin treatment in an animal model in which some degree of artery calcification had been induced by high doses of vitamin D. Treatment with high doses of vitamin D has been known for many years to induce artery calcification in humans, rats, and other animals.^{32–37} This vitamin D-induced calcification is confined to the media of arteries and closely resembles the pattern of calcification seen in Monckeberg's syndrome in humans.^{24,25} In the vitamin D treatment regime used for the current studies, vitamin D induced marked artery calcification within 4 days of treatment.³³ We report here that concurrent Warfarin treatment accelerated artery calcification in the vitamin D-treated rat in this 4-day treatment interval, even though Warfarin treatment alone did not cause detectable artery calcification at treatment times of 1 week or less. The vitamin D/Warfarin model described here has advantages for future studies of the mechanism by which MGP normally inhibits artery calcification, because it is now possible to examine the difference in the nature of the MGP interaction with mineral in a situation in which MGP is γ -carboxylated and mineralization is inhibited and in a situation in which MGP is not γ -carboxylated and mineralization is accelerated.

Methods

Materials

Vitamin K₁ (phyloquinone), vitamin D₃ (cholecalciferol), and Warfarin were purchased from Sigma Chemical Co. For injections, stock solutions of vitamin K₁ were prepared at 10 mg/mL and stored in sterile, foil-wrapped containers at 4°C. Stock solutions of sodium Warfarin were prepared at 50 mg/mL in 0.15 mol/L NaCl and stored in sterile, foil-wrapped containers at 4°C. The stock solution of vitamin D was prepared for subcutaneous injection by dissolving 33 mg of vitamin D₃ (1.32×10^6 IU) in 200 μL of absolute ethanol and mixing this solution with 1.4 mL of emulphor (alkamuls EL-620, Rhone-Poulenc) for 15 minutes. Water (18.4 mL) containing 750 mg of dextrose was then added, and the final solution was mixed for an additional 15 minutes, placed in foil-wrapped containers, and stored at 4°C. Fresh vitamin D solution was prepared for each 3-day injection cycle. Simonsen albino rats (Sprague-Dawley derived) were purchased from Simonsen Labs (Gilroy, Calif).

Methods

For measurement of mineral and MGP accumulation in arteries, each tissue was removed within 30 minutes of death and immediately frozen. Tissues were subsequently washed by continuous mixing with 1 mL of wash solution (100 mmol/L CaCl₂, 20 mmol/L HEPES [pH 7.4], 0.15 mol/L NaCl, and 0.02% NaN₃) for 24 hours at 37°C.

The wash solution was exchanged for fresh solution, and the wash was continued for an additional 24 hours. Washed aortas were briefly patted with a dry tissue and demineralized with 1 mL of 10% formic acid for 24 hours at room temperature. The MGP levels in the acid extracts and in serum were determined by radioimmunoassay as described previously³⁸; this assay uses polyclonal antiserum and reacts identically with native MGP and with MGP in which Glu residues have been heat-decarboxylated to Glu. Calcium levels in acid tissue extracts and serum were determined colorimetrically by using cresolphthalein complexone (Sigma), and phosphate levels were determined colorimetrically as described.³⁹ Tissue sectioning and staining were performed by Biological Testing Service, Inc (Sorrento Valley, Calif).

To purify MGP for determining the γ -carboxylation status of the protein, aortas were dissected at sacrifice from 3 rats treated with 300 000 IU/kg of vitamin D at 0, 24, and 48 hours and with Warfarin every 12 hours, beginning with the first vitamin D injection, for a total Warfarin treatment time of 96 hours. Arteries were rinsed with 20 mmol/L HEPES [pH 7.4], 0.15 mol/L NaCl and dried. The dried aortas were then extracted with 4 changes of 20 mL of 6 mol/L guanidine-HCl with 20 mmol/L HEPES, pH 7.4, for 48 hours at room temperature; rinsed with 20 mmol/L HEPES, pH 7.4, with 0.15 mol/L NaCl and 100 mmol/L CaCl₂; and demineralized for 2 hours with 1.5 mL of 0.15 mol/L HCl at 4°C. A 200- μ L aliquot of the acid extract was then adsorbed to a polyvinylidene difluoride (PVDF) membrane by using a Proisorb device (Perkin-Elmer) and subjected to N-terminal protein sequencing. MGP was also purified from rat bone as described,^{10,40} adsorbed onto PVDF, and sequenced. The degree of γ -carboxylation at the glutamic acid residue at position 2 in the MGP sequence was calculated from a comparison of the recovery of glutamic acid at residue 2 to the value predicted from a repetitive yield analysis of the recovery of amino acids in the first 10 residues of the protein.⁴¹

Maintenance of Animals

Male Sprague-Dawley rats were fed ad libitum with rodent diet 5001 (Purina Mills Inc), a diet that is 0.67% phosphorus and 0.95% calcium by weight. This diet contains 500 μ g/kg of phylloquinone and has no added menadione. All injections were administered subcutaneously into the backs of the animals essentially as described in earlier studies¹⁶. At 24 and 48 hours before the first Warfarin injection, all rats received doses of 1.5 mg vitamin K₁ per 100 g of body weight. Previous studies had shown that this loading dose of vitamin K was necessary to prevent bleeding during the first week of Warfarin treatment.⁴² On the initial day of Warfarin treatment, the first Warfarin dose, 15 mg/100 g of body weight, and 1.5 mg of vitamin K₁ per 100 g, were administered at 8 AM, and the second identical dose of Warfarin was administered at 8 PM with no accompanying vitamin K. This routine was maintained every day until termination of the experiment. Animals were killed by exsanguination while under metofane anesthetic, and selected tissues were removed and fixed in 10% buffered formalin or frozen at -20°C for later studies.

Experiments on the Effects of Age and Growth Status on Warfarin-Induced Artery Calcification

To determine the effect of age on the susceptibility to Warfarin induced artery calcification, male Sprague-Dawley rats were treated with Warfarin beginning at 20 and 42 days of age and at 10 months of age. The 4 animals in each age group received injections of Warfarin every 12 hours and vitamin K every 24 hours (see detailed procedure above) until the end of the experiment. The animals in the 20- and 42-day groups were killed by exsanguination after 2 weeks of Warfarin treatment, and the animals in the 10-month group were killed after 4 weeks of Warfarin treatment. This experiment has been repeated several times with consistent results for animals in each age group.

To evaluate the effect of growth rate on artery calcification in 20-day-old rats, pilot experiments were carried out to establish the measured weight of the diet that allowed maintenance of body weight without allowing bone growth (as evidenced by a failure to increase tibia and femur length). These experiments established 6 g/d

as a suitable maintenance diet. In the first experiment, 12 weanling, male Sprague-Dawley rats, 20 days of age, were caged individually and fed either ad libitum (6 rats) or a measured 6 g/d (6 rats) throughout the experiment. Three rats in each group received Warfarin every 12 hours and vitamin K every 24 hours from the start of the experiment, and the other 3 were not injected. All rats were killed by exsanguination 14 days after the beginning of Warfarin treatment and dietary manipulation.

In the second experiment, 8 male rats 20 days of age were caged individually and fed a measured 6 g/d for 2 weeks. Four of the animals were continued on the 6 g/d diet for an additional 2 weeks, and 4 were placed on an ad libitum diet for an additional 2 weeks. All 8 animals received Warfarin every 12 hours and vitamin K every 24 hours for weeks 3 and 4 only, and all rats were killed 28 days after the start of the experiment, at the end of week 4.

Experiments on the Synergy Between Warfarin and Vitamin D in Artery Calcification

Previous studies on artery calcification in vitamin D-treated rats have used oral gavage to deliver the vitamin.³³ In our first series of experiments, we used this gavage method to examine the effects of Warfarin on artery calcification in rats treated with 300 000 IU/kg of vitamin D at 0, 24, and 48 hours. For subsequent studies, we developed a subcutaneous injection vehicle (see above) for delivering vitamin D to ensure greater uniformity in vitamin D dose. In the initial pilot experiment with the injection method, we examined the effects of Warfarin on rats treated with 300 000 IU/kg of vitamin D at 0, 24, and 48 hours. At the 96-hour time point examined, an identical degree of artery calcification was found for animals treated with vitamin D by the gavage and injection methods, which indicates that vitamin D is taken up to a similar extent from the gut and from the subcutaneous injection site. The subcutaneous injection vehicle developed for the present studies may be useful in future studies of this vitamin in animal models.

In the initial survival study, 24 seven-week-old, male Sprague-Dawley rats received subcutaneous doses of 300 000 IU vitamin D per kg of body weight at 0, 24, and 48 hours. Starting at t=0, 15 of these animals received injections of Warfarin every 12 hours and of vitamin K every 24 hours (see detailed procedure above), and 9 animals received injections of vitamin K alone every 24 hours. Survival was noted every 12 hours.

In the vitamin D dose-response study, 30 seven-week-old, male Sprague-Dawley rats were divided into 5 groups of 6 rats each. Each group was given subcutaneous injections of a different dose of vitamin D (100 000, 200 000, 300 000, or 500 000 IU vitamin D per kg) or of vehicle at t=0, 24, and 48 hours. Starting at t=0, half of the animals in each group received injections of Warfarin every 12 hours and of vitamin K every 24 hours, and the remaining animals in each group received injections of vitamin K alone every 24 hours. The 3 Warfarin-treated rats in the 500 000 IU vitamin D group died between 72 and 84 hours. All surviving animals were killed by exsanguination at 96 hours.

In the time-course study, 24 seven-week-old, male Sprague-Dawley rats received subcutaneous doses of 300 000 IU vitamin D per kg at t=0, 24, and 48 hours. Starting at t=0, 12 of these animals received injections of Warfarin every 12 hours and of vitamin K every 24 hours, and 12 animals received injections of vitamin K alone every 24 hours. Two animals from each group were killed at 48 hours, 4 from each group at 72 hours, and 6 from each group at 96 hours. All animal experiments were approved by the University of California at San Diego animal subjects committee.

Results

Effect of Age on Artery Calcification in Warfarin-Treated Rats

Age has a dramatic effect on artery calcification in the Warfarin-treated rat. As shown in Figure 1, 20-day-old rats treated with Warfarin for 2 weeks had a similar focal pattern of aortic calcification to that seen earlier in 42-day-old rats treated with Warfarin for 2 weeks.¹⁶ The primary differences

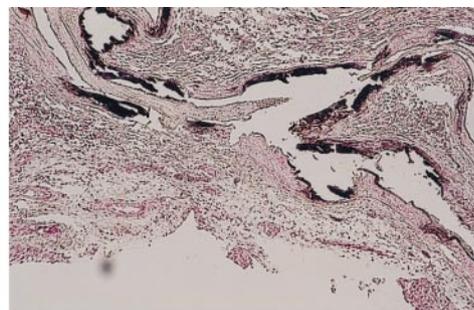
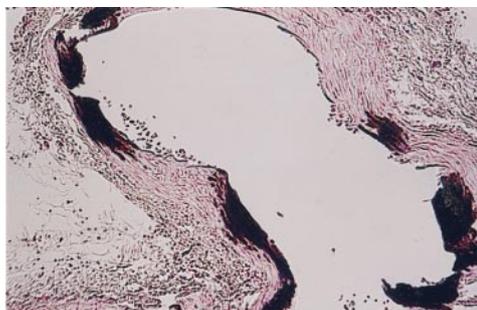
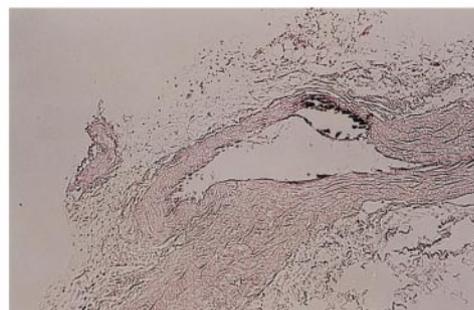
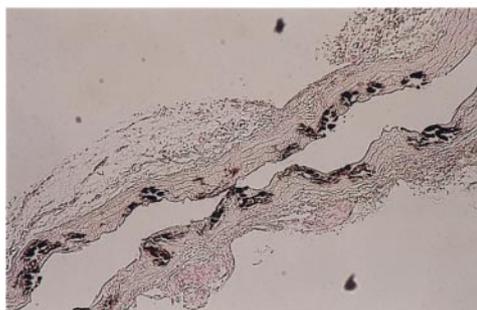
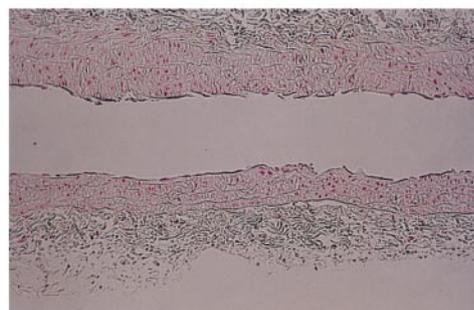
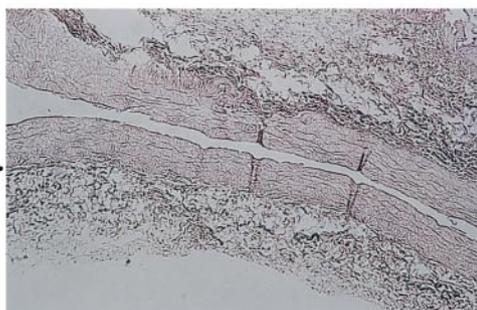
**20-day-old rats after
2 weeks on Warfarin****42-day-old rats after
2 weeks on Warfarin****10-month-old rats after
4 weeks on Warfarin**

Figure 1. Effect of Warfarin treatment on aortic calcification in 20-day-old, 42-day-old, and 10-month-old rats. Male Sprague-Dawley rats were treated with Warfarin every 12 hours and with vitamin K every 24 hours for periods of 2 weeks (20- and 42-day-old animals) and 4 weeks (10-month-old animals). The abdominal aorta segment between the renal branch and the femoral bifurcation was removed immediately at death from the 4 Warfarin-treated animals in each age group and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa's stain. The figure illustrates the typical level of calcification seen in the aorta from 2 animals in each age group. No calcification could be detected in untreated control animals at these ages (not shown).

between the 20- and 42-day-old animals are that the younger animals had a greater number of areas with medial calcification and the intensity of von Kossa staining in these areas was more intense. In contrast, 10-month-old rats proved to be remarkably resistant to Warfarin-induced artery calcification, with no detectable von Kossa staining in the aorta (Figure 1), carotid artery, or aortic heart valves after 4 weeks of Warfarin treatment.

Serum calcium, phosphate, and MGP levels are shown in Table 1 for 20-day-old, 42-day-old, and 10-month-old Warfarin-treated rats. Serum phosphate levels were similar for 20- and 42-day-old rats but are significantly lower for the 10-month-old rats ($P<0.001$). Serum calcium, phosphate, and MGP levels were also determined in age-matched, vitamin K-replete control rats (data not shown). These measurements showed that Warfarin treatment did not affect serum calcium and phosphate levels at any age but did reduce serum MGP levels by 3-fold in the 20- and 42-day-old rats and by 2.5-fold in the 10-month-old rats. A similar reduction in circulating levels of MGP has been seen previously in Warfarin-treated rats, but it is presently unknown whether the lower levels of this serum protein are due to increased

clearance from the blood or decreased release into the bloodstream from sites of MGP synthesis.

Effect of Growth on Warfarin-Induced Artery Calcification in 20-Day-Old Rats

To separate the effects of age and growth on the susceptibility to calcification in the Warfarin-treated rat, 20-day-old rats were placed on the same diet and fed either ad libitum or 6

TABLE 1. Effect of Age on Serum Levels of Calcium, Phosphorus, and MGP in Warfarin-Treated Rats

Age at Start of Warfarin Treatment	mg Ca/dL Serum	mg P/dL Serum	ng MGP/mL Serum
20-Day-old	11.6±0.5	11.4±0.8	84±30
42-Day-old	11.8±0.3	11.0±0.6	60±7
10-Month-old	10.9±0.5	8.0±1.2*	99±21

Rats of the indicated age were treated with Warfarin plus vitamin K for 2 weeks (20- and 42-day-old) or 4 weeks (10-month-old), and serum levels of calcium, phosphorus, and MGP were determined as described in Methods. Data are mean±SD for the 4 rats in each group.

*Significantly reduced compared with 20-day-old and 42-day-old values, $P<0.001$.

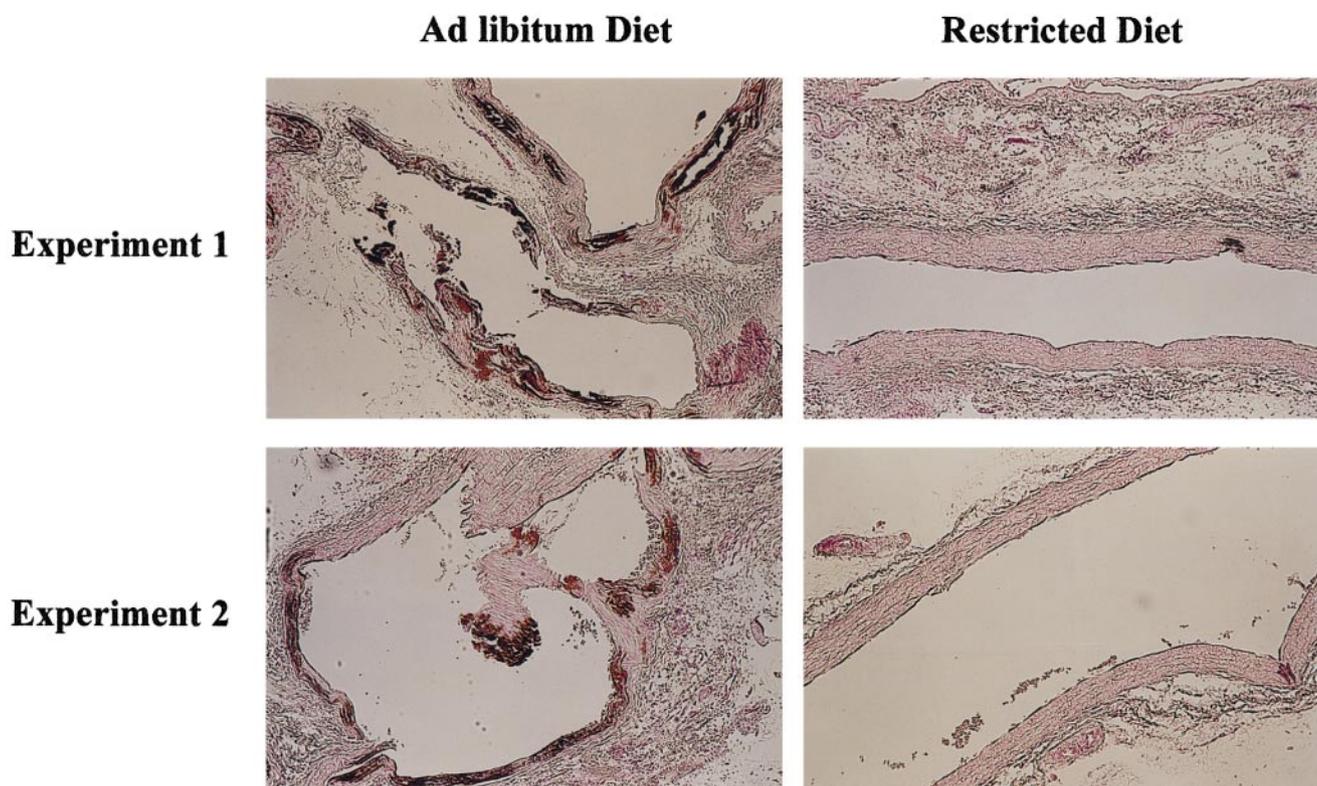


Figure 2. Effect of Warfarin treatment on aortic calcification in 20-day-old rats fed either ad libitum or calorically restricted diets. The abdominal aorta segment between the renal branch and the femoral bifurcation was removed immediately at death from the animals in each dietary treatment group and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa's stain. The figure illustrates the typical level of calcification seen in aortas from 1 animal in the ad libitum diet group and from 1 animal in the 6-g/d diet-restricted group from both experiments 1 and 2. Experiment 1: 20-day-old, male Sprague-Dawley rats were fed a standard rat chow diet either ad libitum (3 animals) or at a measured 6 g/d (3 animals) and were treated concurrently for 2 weeks with Warfarin every 12 hours and vitamin K every 24 hours. Experiment 2: 20-day-old, male Sprague-Dawley rats were fed a measured 6 g/d for 2 weeks. At that time the rats were divided into 2 groups of 4 animals each. One group was continued on the calorically restricted diet for the final 2 weeks and the other group was fed ad libitum for the final 2 weeks. Both groups were treated during the final 2 weeks with Warfarin every 12 hours and vitamin K every 24 hours.

g/d, a restricted food intake that allows maintenance of body mass but permits little or no net growth, as evidenced by the failure of long bones to increase in length. As shown in Figure 2, experiment 1, 2 weeks of Warfarin treatment produced the expected extensive calcification of the artery media in the ad libitum-fed animals. In contrast, no von Kossa staining could be detected in the aorta (Figure 2), carotid arteries, or heart valves of the calorically restricted weanling rats after 2 weeks of Warfarin treatment. Analysis of the acid demineralization extracts of the carotid arteries at the end of the experiment showed that the levels of mineral phosphate and calcium in the ad libitum-fed group were 7-fold above control levels, whereas the levels of mineral phosphate and calcium in the restricted diet-fed rats were at control levels (data not shown). The molar ratio of calcium to phosphate in the carotid arteries of the ad libitum-fed, Warfarin-treated rats was 1.50:1.

Serum calcium, phosphate, and MGP levels were determined at the end of the 2-week period of ad libitum or restricted diet feeding, and the values are shown in Table 2. As shown, rats fed the calorically restricted diet had significantly lower levels of serum phosphate ($P < 0.001$) compared with rats fed ad libitum. Warfarin treatment had no effect on serum calcium and phosphate levels in either the ad libitum-fed or restricted diet groups but did reduce serum MGP levels by >2-fold.

A second growth study was carried out to determine whether rats that were initially fed a calorically restricted diet would become susceptible to Warfarin-induced artery calcification when subsequently fed ad libitum. All 20-day-old

TABLE 2. Effect of Growth Rate and Warfarin Treatment on Serum Levels of Calcium, Phosphorus, and MGP: Experiment 1

Treatment	mg Ca/dL Serum	mg P/dL Serum	ng MGP/mL Serum
Ad libitum diet+Warfarin	11.1±0.1	12.2±1.1	116±12†
Restricted diet+Warfarin	10.3±0.3	8.6±0.7*	61±8†
Ad libitum diet control	11.7±0.2	13.0±0.2	275±26
Restricted diet control	11.0±0.2	9.2±0.3*	232±12

Twenty-day-old male rats were maintained for 2 weeks with the following treatments: ad libitum or calorically restricted diet and treatment with Warfarin plus vitamin K or vitamin K alone. Serum levels of calcium, phosphorus, and MGP were determined as described in Methods. Data are mean±SD for the 3 rats in each group.

*The phosphorus values for all animals on the restricted diet are significantly reduced compared with those of all animals on the ad libitum diet, $P < 0.001$.

†Significantly reduced compared with control animals on the same dietary protocol that did not receive Warfarin, $P < 0.001$.

rats were first fed a calorically restricted diet for 2 weeks and then divided into 2 groups, 1 of which was continued on the restricted diet for 2 weeks and the other of which was placed on the ad libitum diet for 2 weeks. All rats were treated with Warfarin for the last 2 weeks only. The calorically restricted diet used in these studies essentially prevents weight gain in weanling rats, with a weight gain of only 3% in the final 2 weeks of feeding with this diet. In marked contrast, the rats that were first fed the restricted diet for 2 weeks and then placed on the ad libitum diet for the final 2 weeks had a 2.7-fold increase in body weight in the final 2 weeks (169% increase in body weight). The effect of treating these animals with Warfarin during this final 2-week period of ad libitum feeding was to induce a similar pattern of aortic calcification (Figure 2, experiment 2) to that seen in the ad libitum-fed, 20-day-old rats treated for 2 weeks with Warfarin (Figures 1 and 2, experiment 1). In contrast, rats maintained on the restricted diet for the full 4 weeks and treated with Warfarin for the final 2 weeks only had no evidence of von Kossa staining in the aorta (Figure 2, experiment 2), carotid arteries, or aortic heart valves.

Serum calcium and phosphate levels were determined at the end of the 4-week experiment. Compared with the 4 rats fed ad libitum for the final 2 weeks of the experiment, the 4 rats fed the restricted diet throughout the 4-week experiment had significantly lower levels of serum phosphate (7.5 ± 1.1 versus 10.8 ± 0.8 mg/dL, $P < 0.005$) and slightly lower levels of serum calcium (10.4 ± 0.2 versus 11.8 ± 0.2 mg/dL).

Effect of Warfarin Treatment on the Survival of Vitamin D-Treated Rats

To explore the possible synergy between vitamin D and Warfarin in artery calcification, we selected a vitamin D dosage regimen that has been shown to induce significant artery calcification without causing death.³³ Rats were treated with 300 000 IU of vitamin D per kg of body weight at 0, 24, and 48 hours and every 12 hours with Warfarin or vehicle, starting with the first vitamin D injection. In agreement with earlier studies,³³ there was no mortality in the rats treated with vitamin D alone (Figure 3). In contrast, all rats treated with Warfarin plus vitamin D died within 9 days. This effect of Warfarin appears to be an exacerbation of the known lethal effects of high vitamin D doses, since previous studies had shown that treatment with 500 000 IU/kg of vitamin D alone at 0, 24, and 48 hours caused a 34% mortality by 120 hours,^{32,33} and even higher vitamin D doses are known to be lethal. The cause of death in rats treated with massive doses of vitamin D alone was not established in earlier studies, and we could not establish the cause of death in rats treated with lower vitamin D doses together with Warfarin. We noted a marked 15% to 20% weight loss in the period preceding death. This weight loss, which has been noted previously in rats treated with lethal doses of vitamin D alone,³⁵ may be a factor in mortality, but the cause of this weight loss is unknown. It should be noted that the mortality observed in rats treated with vitamin D plus Warfarin is not due to an effect of Warfarin on blood coagulation, since coagulation times remained normal throughout the time course of treatment with vitamin D and Warfarin, and there was no evidence of bleeding in any of the animals.

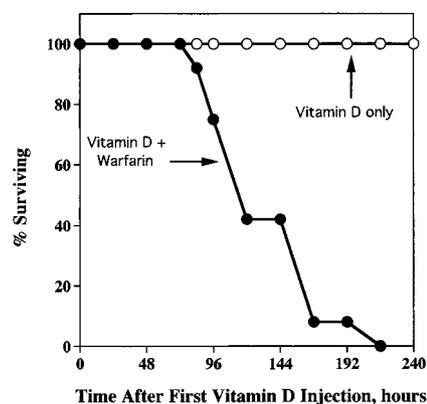


Figure 3. Effect of Warfarin treatment on the survival of vitamin D-treated rats. Seven-week-old, male Sprague-Dawley rats were given subcutaneous injections of 300 000 IU vitamin D/kg body weight at $t=0$, 24, and 48 hours. Beginning with the first vitamin D injection, the 12 experimental rats were treated with subcutaneous injections of vitamin K every 24 hours and Warfarin every 12 hours, and the 9 control rats were treated with injections of vitamin K alone every 24 hours (See Methods for details). The percent of animals surviving is plotted against time from the first vitamin D injection for the Warfarin-treated rats (solid circles) and for the vitamin K-replete rats (open circles).

Effect of Warfarin on Artery Calcification in Vitamin D-Treated Rats

To establish the effect of concurrent Warfarin treatment on artery calcification in the vitamin D-treated rat, rats were treated with the 300 000 IU/kg dose of vitamin D at 0, 24, and 48 hours and every 12 hours with Warfarin or vehicle for 96 hours beginning with the first vitamin D injection. In agreement with earlier studies, treatment with vitamin D plus vehicle caused calcification of the media of the aorta (Figure 4), carotid, and other arteries. Warfarin treatment dramatically increased the extent of medial calcification in the aorta (Figure 4) and other arteries compared with the calcification seen in rats treated with vitamin D alone, but there was no evidence for a Warfarin effect on medial thickness. In agreement with earlier studies, there was no evidence of von Kossa staining of mineral in the arteries of rats treated for 96 hours with Warfarin alone (not shown). In rats treated with vitamin D plus Warfarin, calcification appeared to occur throughout individual, circumferential, lamellar sheets of elastin, resulting in a ribbon-like histological pattern of von Kossa staining for mineral (Figure 4). Hematoxylin/eosin staining of adjacent sections of arteries from the rats treated with vitamin D alone or with vitamin D plus Warfarin revealed no evidence of necrosis in cells in the artery media, either adjacent to sites of intense calcification or in uncalcified regions. This observation indicates that artery calcification in rats treated with vitamin D plus Warfarin occurs in the context of apparently healthy arterial cells and is not initiated by local cell necrosis.

To establish the dependence of artery calcification on the dose of vitamin D, rats were treated with different doses of vitamin D at 0, 24, and 48 hours and treated every 12 hours with Warfarin or vehicle beginning with the first vitamin D injection. As shown in Figure 5, the level of phosphate in the acid demineralization extract of the aorta and carotid artery increased with increasing vitamin D dose, and Warfarin treatment markedly enhanced the extent of phosphate accu-

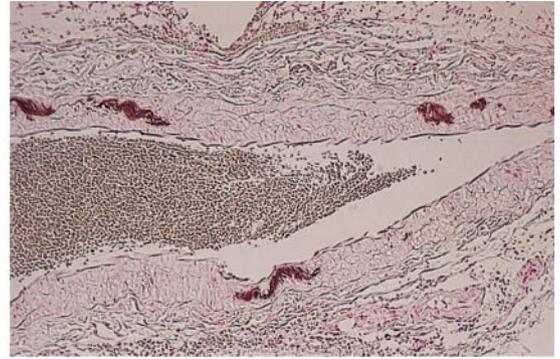
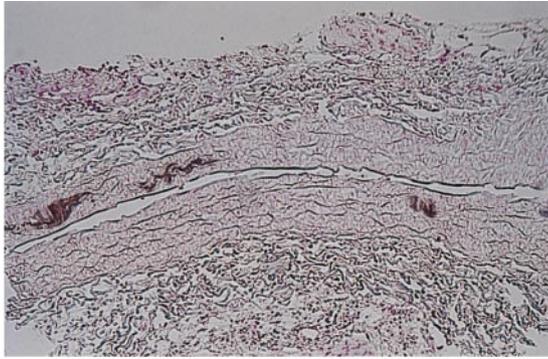
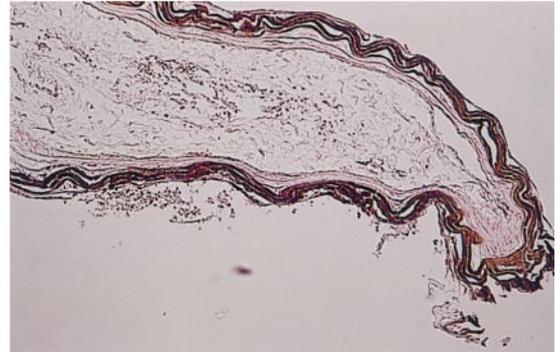
Vitamin D**Vitamin D
+ Warfarin**

Figure 4. Effect of Warfarin treatment on aortic calcification in vitamin D–treated rats. Seven-week-old, male Sprague-Dawley rats were given subcutaneous injections of 300 000 IU vitamin D/kg body weight at $t=0$, 24, and 48 hours. Beginning with the first vitamin D injection, the 4 experimental rats were treated with subcutaneous injections of vitamin K every 24 hours and Warfarin every 12 hours, and the 4 control rats were treated with injections of vitamin K alone every 24 hours (See Methods for details). The animals were killed 96 hours after the first vitamin D injection, the abdominal aorta segment between the renal branch and the femoral bifurcation was removed from each animal at necropsy and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa's stain. The figure illustrates typical sections from 2 rats treated with vitamin D plus Warfarin (bottom) and from 2 rats treated with vitamin D only (top).

mulation at each vitamin D dose. As also shown in Figure 5, Warfarin treatment did not cause a significant accumulation of phosphate in acid demineralization extracts of the artery in the absence of concurrent vitamin D treatment (see results in Figure 5 for the zero dose of vitamin D); this result is consistent with previous studies that showed that treatment with Warfarin alone did not cause detectable artery calcification until 2 weeks of treatment.¹⁶ Calcium levels were also determined in each acid demineralization extract, and the effect of vitamin D dose on calcium accumulation paralleled the effect on phosphate accumulation, with an average calcium-to-phosphate ratio in the acid extracts of 1.46 to 1.50:1 (data not shown).

The effect of vitamin D and Warfarin treatment on the accumulation of MGP in the artery was determined by measurement of MGP levels in each acid extract by radioimmunoassay. As shown in Figure 6, MGP levels increased with vitamin D dose, and the MGP levels in rats treated with vitamin D plus Warfarin were markedly higher than those in rats treated with the same dose of vitamin D alone. The accumulation of MGP in the artery exactly paralleled the extent of accumulation of phosphate and calcium, and the ratio of MGP level to the amount of calcium phosphate mineral in artery extracts was the same at different levels of total calcification in animals within the Warfarin plus vitamin D group and in animals within the vitamin D only group. The ratio for the Warfarin-treated animals was, however, $\approx 50\%$ lower than that in the vitamin D–only group; this result is consistent with previous report that Warfarin also

reduces MGP accumulation in bone by 50%.¹⁶ To determine the effect of Warfarin treatment on the γ -carboxylation of MGP, it was purified from the acid extract of aortas from rats treated with 300 000 IU/kg of vitamin D at 0, 24, and 48 hours and every 12 hours with Warfarin, beginning with the first vitamin D injection. *N*-Terminal protein sequencing was then used to measure the degree of γ -carboxylation at residue 2 in the protein. The MGP that accumulated in the calcified aorta of rats treated with vitamin D plus Warfarin was less than 5% carboxylated at residue 2, compared with $>96\%$ carboxylation at residue 2 for MGP isolated from normal rat bone. This result demonstrates that Warfarin treatment does inhibit γ -carboxylation of MGP, which accumulates in arteries with calcification, and that the accumulation of MGP in these arteries must therefore not be dependent on the γ -carboxylation status of the protein.

The vitamin D doses that induce artery calcification substantially elevate serum calcium levels compared with the 11.5 mg/dL level in control rats, with a 29% increase at 100 000 IU/kg and a 40% increase at 200 000, 300 000, and 500 000 IU/kg (data not shown). Concurrent treatment with Warfarin did not change serum calcium levels compared with those in rats treated with vitamin D alone. Serum phosphate levels were not significantly changed by any dose of vitamin D or by concurrent treatment with Warfarin (data not shown). These results indicate that elevated serum calcium levels could contribute to the calcification of arteries in rats treated with vitamin D alone, but that the synergistic effect of Warfarin on artery calcification cannot be explained by an effect of Warfarin on serum calcium or phosphate levels.

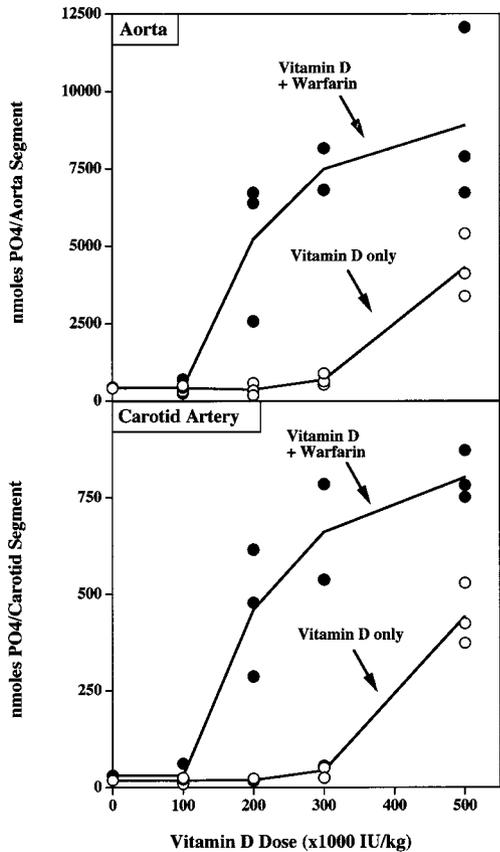


Figure 5. Mineral accumulation in aortas of rats treated with different doses of vitamin D and either Warfarin or vehicle. Thirty 7-week-old, male Sprague-Dawley rats were divided into 5 groups of 6 rats each, and the groups were given subcutaneous injections of vehicle (vitamin D dose=0) or of 100 000, 200 000, 300 000, or 500 000 IU vitamin D/kg body weight at t=0, 24, and 48 hours. Beginning with the first vitamin D injection, half of the rats in each group were treated with subcutaneous injections of vitamin K every 24 hours and Warfarin every 12 hours and the other half with injections of vitamin K alone every 24 hours. The 3 animals treated with Warfarin plus the 500 000-IU dose of vitamin D died between 72 and 84 hours, and all other animals were killed at 96 hours. The aorta and carotid arteries were removed and extracted with acid, and the acid extract was analyzed for phosphate. Total phosphate levels in the acid extracts of aorta and carotid arteries from each animal are plotted (1 point per animal) for rats treated with Warfarin plus the given dose of vitamin D (closed circles) and for rats treated with this dose of vitamin D alone (open circles; see experimental procedures for details). Top, Aorta segment from the aortic arch to the femoral bifurcation. Bottom, Carotid artery segment from the aortic arch to the bifurcation of the interior and exterior carotid branches.

Time Course of Artery Calcification in Rats Treated With Vitamin D and Warfarin

To determine the time course of artery calcification, rats were treated with the 300 000 IU/kg dose of vitamin D at 0, 24, and 48 hours together with Warfarin or vehicle for a total treatment interval of 48, 72, or 96 hours. As shown in Figure 7, the total level of mineral phosphate in the acid extract of the aortas was increased markedly at 96 hours in rats treated with Warfarin plus vitamin D compared with rats treated with vitamin D alone. Similar results were obtained for calcium levels in the acid extracts, and the calcium-to-phosphate molar ratios in the acid extract were 1.50:1. As noted above (Figure 5), treatment for 96 hours with Warfarin alone did not

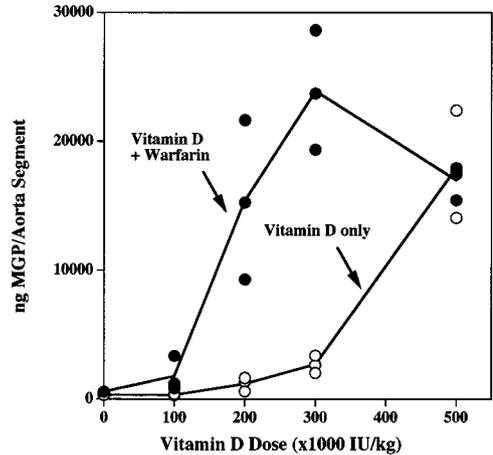


Figure 6. MGP accumulation in the aorta of rats treated with different doses of vitamin D and either Warfarin or vehicle. Total MGP levels in each aorta acid extract from the experiment shown in Figure 5 were determined by radioimmunoassay (see experimental procedures for details). Closed circles (1 per animal) indicate rats treated with Warfarin plus the indicated dose of vitamin D; open circles, rats treated with the indicated dose of vitamin D alone.

cause a significant accumulation of calcium or phosphate in the artery. Serum calcium levels were significantly elevated by vitamin D treatment at 48, 72, and 96 hours (Figure 8), and concurrent treatment with Warfarin again did not affect serum calcium levels compared with rats treated with vitamin D alone.

Discussion

Warfarin-Induced Artery Calcification Is Accelerated by Growth

We previously examined the effect of Warfarin on artery calcification in 42-day-old rats and found that Warfarin

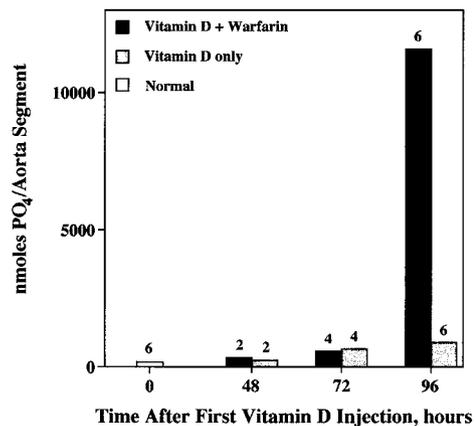


Figure 7. Time course of mineral accumulation in the aorta of rats treated with Warfarin plus vitamin D or with vitamin D only. Seven-week-old, male Sprague-Dawley rats were given subcutaneous injections of 300 000 IU of vitamin D/kg body weight at t=0, 24, and 48 hours. Beginning with the first vitamin D injection, rats were treated either with subcutaneous injections of vitamin K every 24 hours and Warfarin every 12 hours (solid columns) or with injections of vitamin K alone every 24 hours (gray columns). Column height is the mean level of phosphate in the acid extract of the aorta obtained from the number of rats indicated at the column top (see experimental procedures for details). The white column (at t=0) is the mean level of phosphate in the acid extract of the aorta obtained from 6 untreated, age-matched control rats.

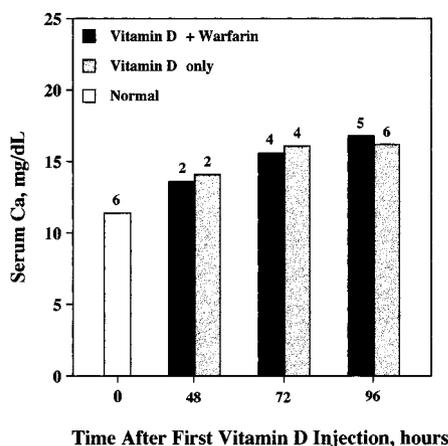


Figure 8. Time course of serum calcium levels in rats treated with Warfarin plus vitamin D or with vitamin D only. Calcium levels were measured in the sera of animals from the experiment shown in Figure 7. Solid columns indicate rats treated with Warfarin plus vitamin D; gray columns, rats treated with vitamin D only; and white column, untreated control rats. Column height is the mean serum calcium level for each group. The number of rats per group is given at the column top.

caused dramatic, focal calcification of the artery media within 2 weeks of treatment.¹⁶ The present studies demonstrate that 20-day-old rats are even more sensitive to Warfarin-induced artery calcification and that 10-month-old rats are completely resistant. One possible explanation for the resistance of the older rat to Warfarin-induced artery calcification could be differences in the sensitivity of the older animal to Warfarin. This drug is cleared rapidly in the rat, with a half-time of 3 to 5 hours in young animals.^{44,45} The rapid clearance of Warfarin in fact necessitated the use of an every-12-hour injection schedule to induce artery calcification in the earlier studies of 42-day-old rats.¹⁶ Even small changes in Warfarin metabolism with age could affect the persistence of the drug over the 12 hour injection cycle and so compromise the effect of Warfarin in the older animal.

In the present investigations, we used diet restriction to induce reversible growth arrest in young rats and so dissociate the effects of growth and age on the susceptibility of arteries to Warfarin-induced calcification. These studies clearly demonstrate that it is rapid growth itself that determines susceptibility to Warfarin-induced calcification, not the age of the animal per se. This observation makes it unlikely that the resistance of older rats to Warfarin-induced artery calcification can be explained by age-related changes in Warfarin metabolism or by other age-related changes in the sensitivity of MGP γ -carboxylation to this dose of Warfarin.

Although there are no previous *in vivo* studies on the effects of age and growth on the extent of calcification in arteries and other soft tissues, the effect of recipient age on the extent of implant calcification has been investigated in 2 earlier studies. Calcification of porcine valves in humans has been modeled by implanting porcine valves at subcutaneous sites in the rat, and in the course of such studies, it was found that implant calcification is more rapid in young animals than in old.⁴⁶ Aortic valves are composed largely of elastin and are therefore similar in composition to the artery media; thus, they may be susceptible to the same calcification-initiation mechanisms as in the rat. In other studies, alkaline phosphata-

se-impregnated collagen particles were implanted subcutaneously into rats of different age. Implant calcification was higher in young rats than in old, and the extent of implant calcification was closely correlated with serum phosphate levels.⁴⁷

It is unclear how growth increases susceptibility to artery calcification in the Warfarin-treated rat. One possibility is that a growing skeleton sheds mineral nuclei, which lodge in the weblike structure of the elastic lamellae of the arterial walls and that, in the absence of avid neutralization of these nuclei by active MGP, mineralization spreads rapidly through the lamellae. Another possibility is that growth acts to promote artery calcification indirectly, through its effect on serum levels of calcium and phosphate. This possibility is supported by the association between serum phosphate and the tendency of Warfarin treatment to induce artery calcification, with the lowest serum phosphate levels in the 2 instances of resistance to Warfarin-induced artery calcification, the 10-month-old rat and the calorically restricted rat (Tables 1 and 2). In humans, the importance of serum calcium and phosphate levels as possible determinants of susceptibility to artery calcification is supported by the association between marked elevations in serum phosphate and extensive artery and soft tissue calcification in renal disease^{48–50} and the association between hypercalcemia and artery and soft tissue calcification in other diseases.⁵¹ Finally, the relationship between growth and Warfarin-induced artery calcification could reflect an effect of growth on other factors in the artery that regulate the calcification process, such as altered expression of growth factors in the arterial wall. We cannot presently rule out any of these possible mechanisms as contributors to the effect of growth on Warfarin-induced artery calcification, and studies are now in progress to further understand the physiological basis for the effect of growth status on susceptibility to artery calcification.

Warfarin and Vitamin D Act Synergistically to Rapidly Calcify Arteries

The present studies demonstrate that a Warfarin dose regime that causes focal artery calcification in rats after 2 weeks of treatment, but not at 1, will increase the calcification of arteries in rats treated with high doses of vitamin D in as little as 3 to 4 days compared with the calcification of arteries seen in rats treated with vitamin D alone. The major difference between the pattern of artery calcification in rats treated with Warfarin plus vitamin D compared with animals treated with the same dose of vitamin D alone, as depicted in Figure 4, is the continuous nature of elastic lamellae calcification in the arteries of the Warfarin plus vitamin D group compared with a more localized calcification in rats treated with vitamin D alone. This pattern suggests that Warfarin treatment has allowed the growth of a greater number of mineralization nuclei within the elastic lamellae. It is noteworthy that the earliest stages of arterial medial calcification in this model often spare the regions between the elastic lamellae, regions occupied by the vascular smooth muscle cells. While we were unable to examine artery calcification in rats treated with Warfarin plus vitamin D at periods of 1 week or longer owing to the lethal nature of the treatment (Figure 3), we examined the longer-term outcome of artery calcification in rats treated with the 300 000-IU dose of vitamin D alone. By 2 weeks,

rats treated with this dose of vitamin D at 0, 24, and 48 hours had solid calcification of all regions of the arterial wall, and the wall itself was rigid (not shown). This result indicates that even in the vitamin K-replete rat, the calcification initiated by the first 3 days of vitamin D treatment progressed without restraint until the entire limits of the artery media had been engulfed by mineral. The critical Warfarin-sensitive mechanism for inhibiting calcification therefore acts early in the artery calcification process and may be largely ineffective once the calcification has reached a massive threshold.

Artery calcification in the vitamin D-treated rat is accompanied by a dramatic accumulation of MGP, and the amount of MGP in the artery is directly proportional to the amount of mineral. MGP also accumulates in proportion to mineral in rats treated with vitamin D plus Warfarin, although that amount of MGP per mg calcium or phosphate is about half as great. A comparable accumulation of MGP per unit mineral was previously found in the slower calcification of arteries induced by this dose of Warfarin alone.¹⁶ Because of the more massive artery calcification in the Warfarin plus vitamin D-treated rat, we were able to isolate sufficient MGP in the current studies to establish that the protein is indeed not γ -carboxylated. We can therefore conclude that γ -carboxylation of MGP is not required for its accumulation at sites of calcification in the arterial wall and that the defect in the activity of MGP as a calcification inhibitor, which accounts for the acceleration of calcification in the Warfarin plus vitamin D-treated rat, must involve the detailed nature of the complex formed between the protein and the mineral and not the simple accumulation of the protein on the mineral surface alone.

The vitamin D doses that cause artery calcification are also those that cause an elevation in serum calcium levels, and the time course of serum calcium elevation is correlated with the onset of artery calcification. A comparable hypercalcemia has been seen in earlier studies of rats treated with high doses of vitamin D.⁵² The close parallel between hypercalcemia and artery calcification seen in the current study suggests that artery calcification in the vitamin D-treated rat may be a simple physical chemical consequence of hypercalcemia. This hypothesis is supported by the fact that hypercalcemia has been previously associated with artery calcification in humans and other animals.⁵¹ It should be noted that the active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃, has been shown to stimulate the expression of MGP in osteoblastic cells in culture.^{5,53} This effect has not been observed in vascular smooth muscle cells and fibroblasts (P.A.P. et al, unpublished observations, 1992), and there is no evidence of increased MGP expression in the artery during the first 24 hours of vitamin D treatment before the onset of calcification. It therefore seems unlikely that vitamin D affects artery calcification through its ability to directly regulate MGP expression by vascular cells.

The Warfarin plus vitamin D treatment model described here has advantages for future investigations of MGP function in the inhibition of artery calcification. Because in this model Warfarin accelerates calcification in a system wherein calcification occurs even in the absence of Warfarin treatment, it should be possible to examine the difference in the nature of the MGP interaction with mineral in a situation in which MGP is γ -carboxylated and mineralization is inhibited

and in a situation in which MGP is not γ -carboxylated and mineralization is accelerated. A second advantage of the Warfarin plus vitamin D model is the rapid onset of calcification, since it should be possible to evaluate the ability of a single MGP injection to arrest early stages of artery calcification in this system.

The major conclusion of these studies is that Warfarin-induced artery calcification is accelerated by growth and by vitamin D. There are at least 2 reasonable hypotheses to account for these observations: (1) Warfarin-induced artery calcification could be promoted by increases in serum calcium or phosphate. This hypothesis is supported by the high levels of serum phosphate in growing rats and the high levels of serum calcium in vitamin D-treated rats. (2) Artery calcification could be promoted by metabolic processes that are activated by growth and by vitamin D. For example, growth and vitamin D both increase bone metabolism, and a bone metabolic process could release mineral nuclei into serum. These nuclei could subsequently lodge in the elastic lamellae of the artery media, where they grow rapidly because of the Warfarin-induced inactivation of the calcification inhibitory activity of MGP. Studies are in progress to establish which of these 2 hypotheses best accounts for the effects of growth and vitamin D on artery calcification.

Acknowledgments

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