Bisphosphonates Alendronate and Ibandronate Inhibit Artery Calcification at Doses Comparable to Those That Inhibit Bone Resorption

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Abstract—The present experiments were carried out to test the hypothesis that artery calcification is linked to bone resorption by determining whether the selective inhibition of bone resorption with the bisphosphonates alendronate and ibandronate will inhibit artery calcification. Artery calcification was first induced by treatment of 42-day-old male rats with warfarin, a procedure that inhibits the γ-carboxylation of matrix Gla protein and has been shown to cause extensive calcification of the artery media within 2 weeks. These experiments revealed that ibandronate (0.05 $mg \cdot kg^{-1} \cdot d^{-1}$) and alendronate (0.1 $mg \cdot kg^{-1} \cdot d^{-1}$) completely inhibited calcification of all arteries and heart valves examined after 2 and 4 weeks of warfarin treatment. A 10-fold lower dose of alendronate reduced artery calcification by 50% ($P<0.005$). These bisphosphonate doses are comparable to those that inhibit bone resorption in rats of this age. More rapid artery calcification was induced by treatment with warfarin together with high doses of vitamin D, a procedure that causes extensive artery calcification by 84 hours. Alendronate and ibandronate again completely inhibited calcification of all arteries and heart valves examined. The subcutaneous doses of alendronate and ibandronate necessary to inhibit artery calcification are comparable to the daily subcutaneous doses of these drugs that have previously been shown to inhibit bone resorption in rats of the same age, with 50% inhibition of artery calcification at 20 μg alendronate $\cdot kg^{-1} \cdot d^{-1}$ and at 1 μg ibandronate $\cdot kg^{-1} \cdot d^{-1}$. Bisphosphonate treatment did not affect serum calcium and phosphate, and so the inhibition of artery calcification cannot be due to a simple lowering of the serum calcium phosphate ion product. We conclude that bisphosphonates inhibit the calcification of arteries and heart valves at doses comparable to the doses that inhibit bone resorption. These results support the hypothesis that artery calcification is linked to bone resorption. The mechanism of this linkage remains to be established, however, and an alternative explanation for the present results is also considered. **(*Arterioscler Thromb Vasc Biol*. 2001;21:817-824.)**

Key Words: artery calcification ■ bisphosphonates ■ bone resorption ■ alendronate ■ ibandronate

The present experiments were carried out to test the hypothesis that artery calcification is linked to bone resorption. This hypothesis originated in experiments we carried out to understand the factors that enhance artery calcification in rats treated with high doses of warfarin, a vitamin K antagonist that inhibits the γ-carboxylation of matrix Gla protein (MGP) and thereby causes extensive calcification of the elastic lamella in the artery media and in heart valves,[1] a pattern of calcification also seen in the MGP gene knockout mouse.[2] In the course of these studies, we observed that warfarin treatment induces artery calcification to the greatest extent in young, rapidly growing rats and that adult rats are completely resistant to warfarin-induced artery calcification.[3] The susceptibility of young rats to warfarin-induced artery calcification is related to growth and not age per se, because warfarin treatment fails to induce artery calcification in young rats fed a restricted diet with a caloric content adequate to maintain body weight without permitting bone growth or weight gain. These experiments showed that growth processes promote artery calcification and were consistent with the hypothesis that bone metabolism could in fact be the critical determinant for susceptibility to warfarin-induced artery calcification. In a second series of experiments, we observed that high doses of vitamin D accentuate artery calcification in rats treated with warfarin.[3] Because vitamin D is known to potently stimulate bone resorption, one explanation for the increased susceptibility of vitamin D–treated rats to warfarin-induced artery calcification could be a link between bone resorption and artery calcification.

The hypothesis that artery calcification is linked to bone resorption is further supported by studies of the osteoprotegerin-deficient mouse.[4] Osteoprotegerin is a secreted protein that inhibits osteoclast formation, and osteoprotegerin-deficient mice have a severe, early-onset osteoporosis that is consistent with excessive osteoclastic

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bone resorption. These mice also have an extensive calcification of the media of the aorta and renal arteries that is similar to the medial calcification seen in the warfarin-treated rat and in the MGP-deficient mouse.

In the present studies, based on the hypothesis that artery calcification is linked to bone resorption, we predicted that bisphosphonate doses that inhibit bone resorption will also inhibit artery calcification. Bisphosphonates are a clinically important class of drugs that are currently used to specifically inhibit osteoclastic bone resorption in humans.[5] Early studies showed that etidronate and other first-generation bisphosphonates inhibit normal bone mineralization at doses comparable to the doses that inhibit bone resorption.[6] This inhibition of bone mineralization was evidenced by formation of wide osteoid seams in the metaphysis and by lack of mineralization in the hypertrophic cartilage zone,[6] and it was observed at doses of 5 to 20 mg $P \cdot kg^{-1} \cdot d^{-1}$. Because of the interest in the use of specific inhibitors of bone resorption to treat clinical disorders such as osteoporosis, a large number of bisphosphonates have been synthesized and tested for their efficacy in inhibiting bone resorption at doses that do not inhibit bone mineralization. Among the bisphosphonates currently in clinical use, 2 of the most potent bone resorption inhibitors are alendronate and ibandronate, which are 1000- and 10 000-fold more effective resorption inhibitors than etidronate, respectively.[5,7] Because ibandronate and alendronate do not inhibit normal bone mineralization even at doses of 1 mg $P \cdot kg^{-1} \cdot d^{-1}$,[8,9] which is far above the dose necessary to inhibit bone resorption, it has been possible to use these and other more potent bisphosphonates to inhibit bone resorption in patients without causing the impairment of normal bone mineralization seen with etidronate and other first-generation bisphosphonates.[5,7]

Etidronate, clodronate, and several other first-generation bisphosphonates have previously been shown to inhibit vitamin D–induced artery calcification in the rat.[10–14] Because the doses of these bisphosphonates that are necessary to inhibit artery calcification, $\geq$5 mg $P \cdot kg^{-1} \cdot d^{-1}$, are comparable to the doses that inhibit normal mineralization of bone, it was thought that the inhibition of both mineralization processes occurred by a common physicochemical mechanism in which the bisphosphonate bound to nascent hydroxyapatite crystals and inhibited their growth.[5,7] This hypothesis was supported by the observation that bisphosphonates bind strongly to hydroxyapatite and potently inhibit formation of calcium phosphate mineral phases from supersaturated solutions of calcium and phosphate in vitro.

In the present study, we have for the first time investigated 2 of the newer generation of more potent bisphosphonate inhibitors of bone resorption, alendronate and ibandronate, as inhibitors of artery calcification, using the low doses of these bisphosphonates that have previously been demonstrated to inhibit bone resorption.

Methods

Materials

Vitamin K_1 (phylloquinone), vitamin D_3 (cholecalciferol), and warfarin were purchased from Sigma Chemical Co. Etidronate (Didronel, Proctor and Gamble Pharmaceuticals) and alendronate (Fosamax, Merck and Co, Inc) were purchased from University City Pharmacy, and ibandronate (Bondronat, Boehringer Mannheim) was purchased from Idis World Medicines. Stock solutions of alendronate and etidronate were prepared in 0.15 mol/L NaCl, titrated to pH 7.4 with NaOH, and stored at 4°C. Ibandronate was diluted with 0.15 mol/L NaCl and stored at 4°C. All bisphosphonate doses are stated in milligrams of phosphorus (mg P) so that the molar effectiveness of the drugs can be compared directly, a method that was used in earlier studies.[8,15] The following values were used to convert from actual measured weight of bisphosphonate to mg P for each drug used: alendronate $(Na)(H_2O)_3$, 62 mg P/325 mg drug; etidronate $(Na)_2$, 62 mg P/250 mg drug; and ibandronate $(Na)(H_2O)$, 62 mg P/357 mg drug. Stock solutions of vitamin K_1 were prepared at 10 mg/mL in 7% emulphor (Alkamuls EL-620, Rhodia, Inc), and stock solutions of sodium warfarin were prepared at 50 mg/mL in 0.15 mol/L NaCl; both were stored in sterile, foil-wrapped containers at 4°C. Finally, stock solutions of vitamin D were prepared fresh for each 3-day subcutaneous injection cycle at a concentration of 1.65 mg/mL in 7% emulphor and then placed in foil-wrapped containers and stored at 4°C, as described previously.[3] Simonsen albino rats (Sprague-Dawley–derived) were purchased from Simonsen Laboratories.

Experimental Procedures

Histological analysis of the calcification of arteries and heart valves was carried out on formalin-fixed tissues as described previously.[1,3] Tissues were first placed in formalin within 30 minutes of death and fixed for 24 hours at room temperature. Embedding, sectioning, and von Kossa staining of tissues were carried out by Biomedical Testing Services, Inc. For histological analysis of the abdominal aorta, the entire abdominal section from 1 cm above the renal branch to just beyond the femoral bifurcation was embedded intact in paraffin and cut with a microtome until the section approximately bisected the aorta cylinder. Both artery walls were then carefully examined for von Kossa–stained foci of calcification, and the number of such foci was noted by 2 observers blinded as to treatment. The number of foci counted by the 2 observers on the same artery was identical in 60% of the arteries examined, and in those arteries in which there was a difference in the number of foci counted by the 2 observers, in no instance was the difference in the number of foci >10% of the total foci in that artery. Carotid arteries were cut into cylindrical segments $\approx$3 mm in length, and transverse sections of the resulting 5 cylinders were examined for the presence of von Kossa–stained foci of calcification. Aortic heart valve calcification was examined in transverse sections.

For measurement of the effect of bisphosphonates on bone mineralization, tibias were dissected free of adhering tissue and were radiographed with a Hewlett-Packard 4380N Faxitron x-ray system. The width of unmineralized growth-plate cartilage in the proximal tibia was measured with calipers and contact radiographs that were magnified 30-fold. The width for each animal is the average of 5 width measurements made in the central half of the growth plate, and the coefficient of variation for the within-group variance was 12%. Osteoid accumulation in microtome sections of the proximal tibial metaphysis was analyzed by Movat's pentachrome technique, as described.[9] For biochemical measurement of mineral accumulation in arteries, each tissue was removed within 30 minutes of death and immediately frozen. Tissues were subsequently washed extensively with buffer and extracted with 1 mL of 10% formic acid for 24 hours at room temperature to dissolve mineral, as described.[3] Calcium levels in serum were determined colorimetrically with cresolphthalein complexone (Sigma), and phosphate levels in serum and in formic acid tissue extracts were determined colorimetrically as described.[16]

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 μg/kg phylloquinone and has no added menadione. In all experiments, animals were killed by exsanguination while under metofane anesthetic, and selected tissues were removed immediately and fixed in 10% buffered formalin or frozen at −20°C for later studies. All animal experiments were approved by the UCSD animal subjects committee.

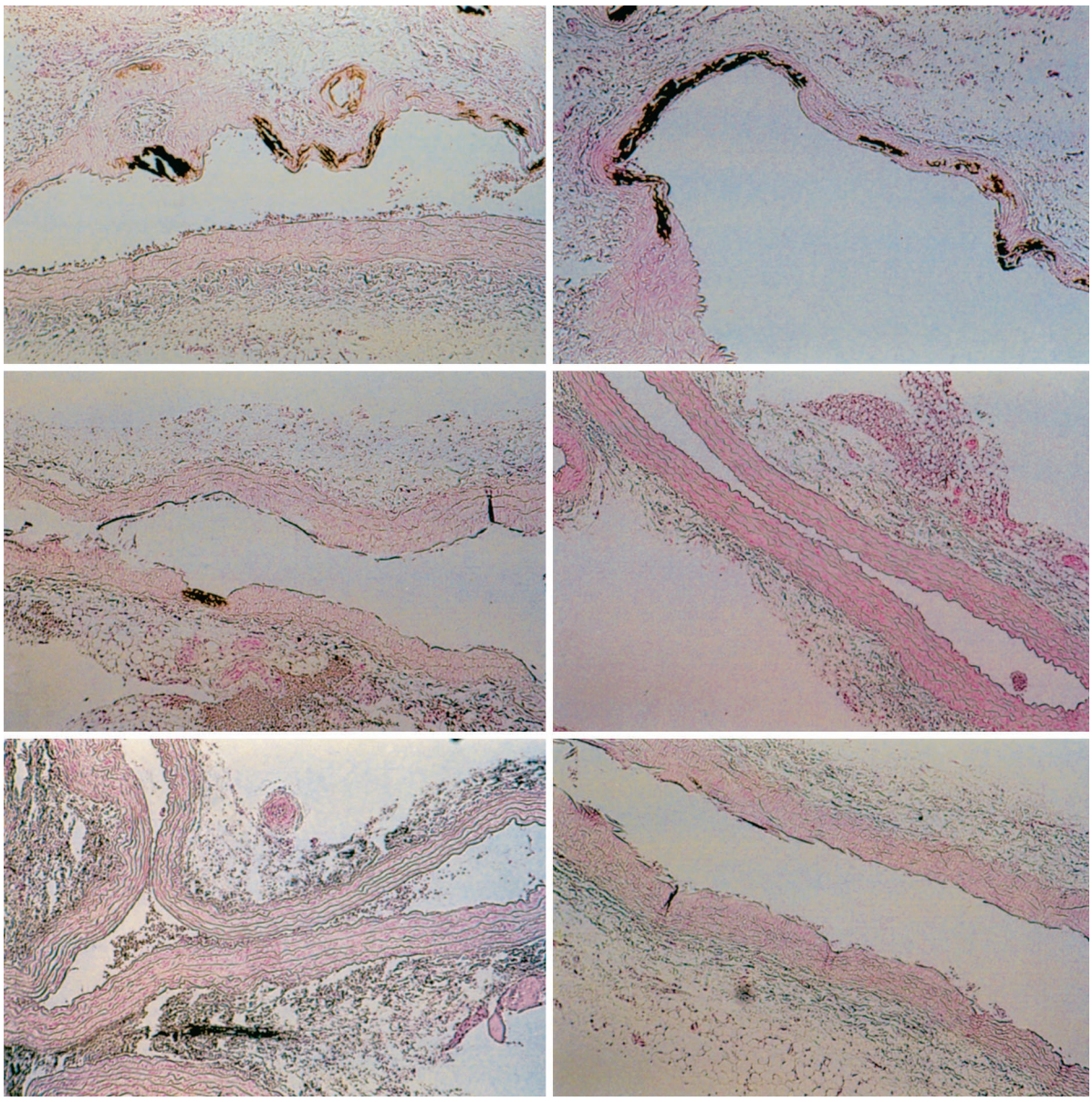

Figure 1. Effect of alendronate and ibandronate on aorta calcification induced by treatment with warfarin for 2 weeks. Twelve 42-day-old male Sprague-Dawley rats were treated daily with warfarin for 2 weeks (see Methods). Starting 4 days before the first warfarin injection, 4 rats received alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, 4 rats received ibandronate at 0.01 mg $P \cdot kg^{-1} \cdot d^{-1}$, and 4 rats received no bisphosphonate. The abdominal aorta was removed immediately after the rats were killed; it was fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa stain. The panels illustrate the typical level of calcification seen in the aorta from 2 animals in each treatment group: top, no bisphosphonate treatment; middle, ibandronate at 0.01 mg $P \cdot kg^{-1} \cdot d^{-1}$; bottom, alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$. No calcification can be detected in untreated control animals at this age.[1]

Effect of Bisphosphonates on Artery Calcification Induced by Warfarin

Rats were treated with warfarin by a procedure[1] that induces detectable artery calcification within 2 weeks without the presence of hypercalcemia. Forty-two-day-old male rats were treated with warfarin for 2 or 4 weeks, and some rats were also treated with daily subcutaneous injections of bisphosphonates beginning 4 days before the first warfarin dose. In the 2-week warfarin treatment experiment shown in Figure 1, 8 rats received no bisphosphonate, 4 rats received alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, and 4 rats received ibandronate at 0.01 mg $P \cdot kg^{-1} \cdot d^{-1}$. In the 4-week warfarin treatment experiment, 8 rats received no bisphosphonate, 4 rats received alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, 4 rats received alendronate at 0.025 mg $P \cdot kg^{-1} \cdot d^{-1}$, and 4 rats received etidronate at 6.25 mg $P \cdot kg^{-1} \cdot d^{-1}$.

Effect of Bisphosphonates on Artery Calcification Induced by Warfarin Plus Vitamin D

Artery calcification was induced by treatment with warfarin plus high doses of vitamin D according to procedures that have been described.[3] In brief, 49-day-old male rats received subcutaneous doses of 300 000 IU vitamin D/kg at t=0, 24, and 48 hours. Where applicable, each animal also received injections of warfarin every 12 hours and of vitamin K every 24 hours starting at t=0 and daily bisphosphonate injections beginning 4 days before the first vitamin D injection. All animals were killed by exsanguination at 84 or 96 hours. In the initial experiment (Figure 2), 6 rats were treated with alendronate at a dose of 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, and 6 rats received no alendronate. In the dose-dependence experiments, animals were treated with the same doses of warfarin, vitamin K, and vitamin D together with the indicated dose and type of bisphosphonate (4 rats per group). In the experiments on the effect of the timing of

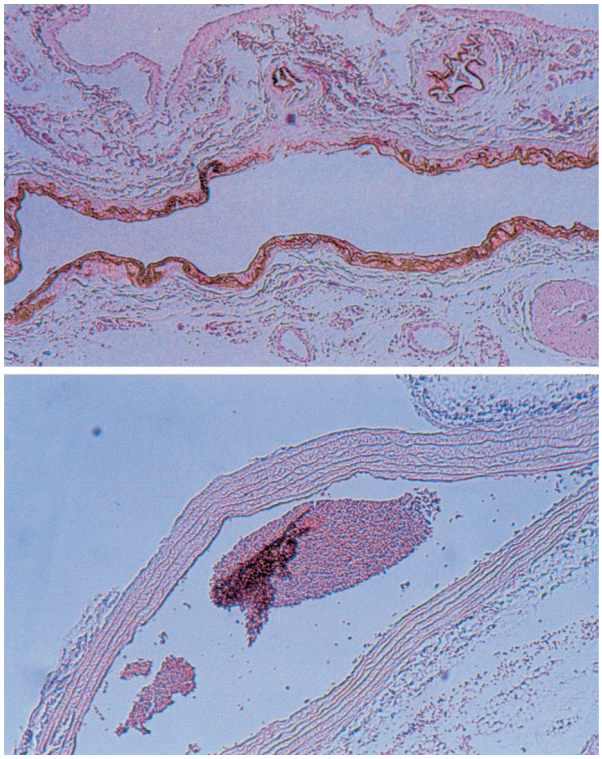

Figure 2. Effect of alendronate on aorta calcification induced by treatment with vitamin D plus warfarin for 96 hours. Twelve 7-week-old male Sprague-Dawley rats were treated with vitamin D and warfarin (see Methods). Six animals were injected subcutaneously with alendronate at a dose of 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ beginning 4 days before the first vitamin D injection, and the remaining 6 animals received no alendronate. All animals were killed 96 hours after the first vitamin D injection; the abdominal aorta was immediately removed from each animal and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral with von Kossa stain. The panels illustrate the typical level of calcification seen in the aorta from 1 animal in each treatment group: top, no alendronate treatment; bottom, alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$.

alendronate administration on artery calcification (Figure 4), animals were again treated with warfarin, vitamin K, and vitamin D together with the following treatment with alendronate at a dose of 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$: One group received no alendronate (11 rats); a second group received alendronate continuously for the entire 8 days, starting 4 days before the first vitamin D injection (6 rats); a third group received alendronate for the first 6 days only, starting 4 days before the first vitamin D treatment and ending with the final dose on the second day of vitamin D treatment (at t=24 hours) (6 rats); and the last group received alendronate only for the last 2 days of the 8-day experiment (at t=48 and 72 hours) (9 rats).

Results

Bisphosphonates Inhibit Artery Calcification Induced by Warfarin

As can be seen in the representative longitudinal sections of the abdominal aorta shown in Figure 1, treatment for 2 weeks with the vitamin K antagonist warfarin induced the same extensive calcification of the artery media as seen in previous studies,[1] and concurrent treatment with the amino bisphosphonates alendronate and ibandronate inhibited this calcification. When the entire length of the abdominal aorta was examined by 2 observers blinded as to treatment, the number of distinct foci of calcification found in the 8 rats treated with warfarin alone ranged from 20 to 47 (mean±SD, 33±9), and

TABLE 1. Effect of Bisphosphonate Treatment on Artery Calcification and on the Proximal Tibial Growth-Plate Width in Warfarin-Treated Rats

Treatment	Carotid PO_4, nmol	Width of Growth-Plate Cartilage, mm
None	49±18	0.31±0.04
Warfarin only	735*±176	0.30±0.04
Warfarin+alendronate at		
0.025 mg $P \cdot kg^{-1} \cdot d^{-1}$	374*†±117	0.29‡±0.04
0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$	63‡±23	0.30‡±0.03
Warfarin+etidronate at		
6.25 mg $P \cdot kg^{-1} \cdot d^{-1}$	55‡±14	2.76*±0.24

Eighteen 42-day-old rats were treated daily with warfarin for 4 weeks. Starting 4 days before the first warfarin injection, 4 rats received alendronate at 0.025 mg $P \cdot kg^{-1} \cdot d^{-1}$, 4 rats received alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, 4 rats received etidronate at 6.25 mg $P \cdot kg^{-1} \cdot d^{-1}$, and 8 rats received no bisphosphonate. Eleven age-matched rats served as untreated controls and received no warfarin or bisphosphonate. The carotid arteries were removed immediately after the rats were killed, and the level of mineral phosphate was determined on the acid extracts of each artery. Both tibias from each animal were removed, cleaned of adhering tissue, and radiographed. The width of the unmineralized growth-plate cartilage was determined for each tibia. (See Methods for details.) The mean±SD values are shown for each group.

*$P<0.001$ vs None.

†$P<0.005$ vs Warfarin only.

‡P=NS vs None.

no foci could be detected in any of the 4 animals treated with warfarin plus alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$. No foci of calcification could be detected in 2 of the 4 rats treated with warfarin plus ibandronate at 0.01 mg $P \cdot kg^{-1} \cdot d^{-1}$; 1 animal had a single small focus of calcification, and the other had 2 small foci. Similar results were found for the carotid arteries and the aortic heart valves from these animals, with 8 to 15 foci in the carotid arteries of the rats treated with warfarin alone and no foci in the carotid arteries of any of the rats treated with warfarin plus alendronate or ibandronate, and with 3 to 8 foci in the elastic lamellae of the aortic heart valves of rats treated with warfarin alone and no foci in heart valves from any of the rats treated with warfarin plus alendronate or ibandronate.

The inhibitory effect of alendronate on warfarin-induced artery calcification was further examined in rats treated for 4 weeks with warfarin, because a longer period of warfarin treatment results in calcification levels that can be measured accurately by chemical analysis of the arteries.[1] As can be seen in Table 1, treatment with warfarin for 4 weeks produced a 15-fold increase in the level of carotid artery calcification, and treatment with warfarin plus 0.25 mg $\cdot kg^{-1} \cdot d^{-1}$ alendronate inhibited this increase. Treatment with the lower alendronate dose of 0.025 mg $P \cdot kg^{-1} \cdot d^{-1}$ inhibited artery calcification by 50%. Identical results were seen on histological analysis of the abdominal aorta from these animals, with numerous, heavily stained foci of calcification in each of the rats treated with warfarin alone, a reduced number of foci in the rats treated with warfarin plus alendronate at a dose of 0.025 mg $P \cdot kg^{-1} \cdot d^{-1}$ ($P<0.01$), and no foci in any of the rats treated with warfarin plus alendronate at a dose of 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ (not shown).

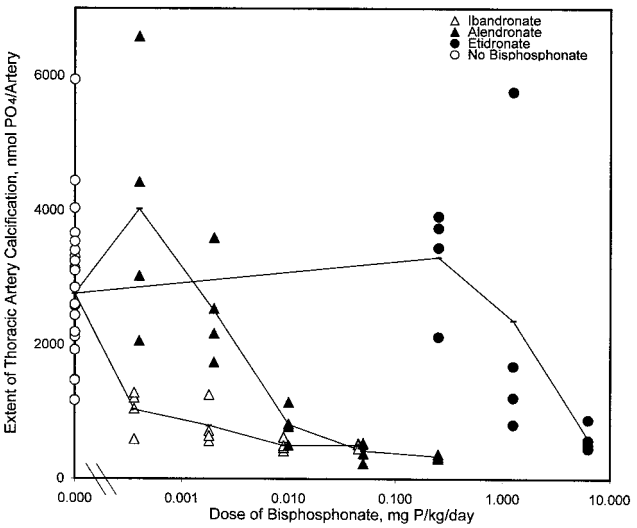

Figure 3. Effect of bisphosphonate type and dose on the extent of mineral phosphate accumulation in the thoracic aorta of rats treated with vitamin D plus warfarin. Seventy 7-week-old male Sprague-Dawley rats were treated with vitamin D and warfarin (see Methods). Twenty-two rats did not receive a bisphosphonate. The remaining 48 rats were divided among 12 treatment groups, and each group was given daily subcutaneous injections of the different bisphosphonates at the indicated doses beginning 4 days before the first vitamin D injection. All animals were killed 84 hours after the first vitamin D injection, and the thoracic aorta segment between the renal branch and the heart was immediately removed from each animal. The level of phosphate in the acid demineralization extract of each artery is shown for all 70 animals, and the lines are drawn to connect the mean values of aorta phosphate in each treatment group. The level of phosphate in the thoracic aorta of untreated control rats of this age is 445±104 (mean±SD) nmol phosphate per thoracic aorta.

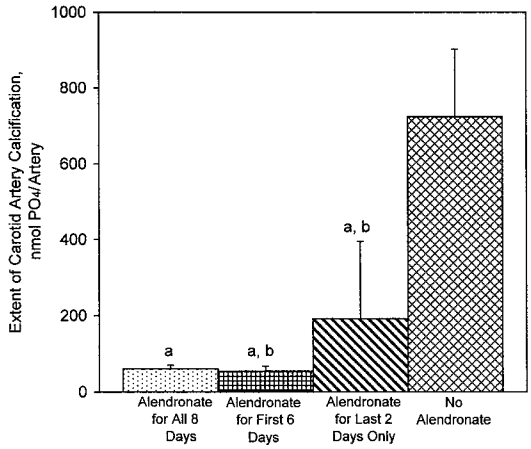

Figure 4. Effect of the timing of alendronate administration on the inhibition of artery calcification in rats treated with vitamin D plus warfarin. Rats were treated with vitamin D and warfarin and were divided into 4 groups based on the timing of alendronate administration (see Methods for details). Eleven animals received no alendronate, 6 received daily alendronate injections continuously for the entire 8 days of the experiment, 6 received alendronate only for the first 6 days of the experiment, and 9 received alendronate for the last 2 days only. All animals were killed 96 hours after the first vitamin D injection. The mean level of phosphate in the carotid artery is shown for each of the 4 treatment groups. The level of phosphate in the carotid artery of untreated control rats of this age is 49±22 (mean±SD) nmol phosphate per carotid artery. a, $P<0.001$ vs No Alendronate. b, P=NS vs Alendronate for all 8 days.

One possible mechanism by which alendronate and ibandronate could affect artery calcification might be to lower serum levels of calcium or phosphate and to thereby reduce the rate at which mineral phases nucleate or grow. We were unable, however, to detect a statistically significant effect of bisphosphonate treatment on serum calcium and phosphate levels measured at the end of the 2- and 4-week warfarin treatment experiments (data not shown).

A second possible mechanism by which alendronate and ibandronate could inhibit artery calcification is by a physicochemical mechanism that involves direct binding of the bisphosphonate with nascent mineral surfaces. This hypothesis predicts that all mineralization processes in an animal will be affected by comparable bisphosphonate doses, and it was advanced to account for the observation that etidronate doses necessary to inhibit vitamin D–induced artery calcification also inhibit normal calcification of bone and cartilage, with the formation of wide osteoid seams and an increase in the width of unmineralized cartilage in the growth plate.[5–7] In agreement with these earlier studies, treatment for 4 weeks with etidronate at a dose of 6.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ inhibited the artery calcification induced by warfarin treatment but also produced a 10-fold increase in the width of unmineralized growth-plate cartilage in the proximal tibia (Table 1) and markedly increased the width of unmineralized osteoid seams in the tibial metaphysis. In contrast, treatment for 4 weeks with alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ inhibited artery calcification but had no effect on the width of unmineralized growth-plate cartilage (Table 1) or on the width of the osteoid seams. There was also no significant effect of treatment with ibandronate or alendronate on the width of unmineralized growth-plate cartilage or unmineralized osteoid seams in the 2-week warfarin treatment experiment described in Figure 1.

Bisphosphonates Inhibit Artery Calcification Induced by Warfarin Plus Vitamin D

The hypothesis that artery calcification is linked to bone resorption was also tested in rats treated with warfarin plus high doses of vitamin D, a procedure that we have shown to cause a far more rapid and extensive calcification of arteries than treatment with warfarin alone, a calcification that can be detected by chemical analysis of arteries after 3 days of treatment.[3] As seen in Figure 2, rats treated for 4 days with warfarin plus vitamin D had extensive von Kossa staining for mineral in their abdominal arteries, whereas rats that also received alendronate at 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ had no evidence of calcification. Chemical analysis of the thoracic aorta and carotid arteries of these animals showed that the 6 animals treated with warfarin plus vitamin D had calcium and phosphate levels 40 to 70 times higher than found in untreated control rats, whereas the 6 animals treated with warfarin, vitamin D, and alendronate had calcium and phosphate levels that were not significantly elevated compared with control rats (data not shown). Histological analyses of the aortic heart valves and kidneys of these animals showed that alendronate treatment also prevented the calcification of these tissues seen in animals treated with warfarin plus vitamin D alone.

We next determined the dose dependence of the effects of alendronate, ibandronate, and etidronate on artery calcifica-

tion induced by treatment with warfarin plus vitamin D so that these could be compared with the doses of these drugs previously found to inhibit bone resorption in rats of this age.[8,17] The dose of each bisphosphonate necessary to reduce the level of artery calcification by half is approximately the same for the thoracic aorta (Figure 3) and the carotid artery (Figure I: published online at http://atvb.ahajournals.org), which shows that the calcifications of these 2 arteries are comparably sensitive to bisphosphonate dose. As is apparent from these 2 figures, there is considerable animal-to-animal variation in the extent of artery calcification in rats treated with vitamin D plus warfarin that is apparent in both the animals that did not receive bisphosphonates and in animals that did. This variation has been noted in earlier studies[3,18] and appears to reflect variability in the short-term effects of high doses of vitamin D on artery calcification. Despite this variation, it is clear that the relative potencies of the 3 bisphosphonates tested as inhibitors of artery calcification are ibandronate $>$ alendronate $>>>$ etidronate. The approximate doses of each bisphosphonate necessary to reduce the level of mineral by half are comparable for the thoracic aorta and the carotid artery and are 0.0002 mg $P \cdot kg^{-1} \cdot d^{-1}$ for ibandronate, 0.005 mg $P \cdot kg^{-1} \cdot d^{-1}$ for alendronate, and 2 mg $P \cdot kg^{-1} \cdot d^{-1}$ for etidronate. The levels of mineral phosphate in the acid demineralization extracts of the thoracic aorta and the carotid arteries at the 2 highest alendronate doses and the 2 highest ibandronate doses were not significantly above control values ($P>0.1$), which were 445 ± 104 (mean$\pm$SD) nmol phosphate per thoracic aorta and 51 ± 22 nmol phosphate per carotid artery.

The abdominal aortas from each of these animals were fixed in formalin, and longitudinal sections were examined for the presence of mineral by 2 observers blinded as to drug treatment. The results of this histological analysis showed that von Kossa staining for mineral was completely eliminated at ibandronate doses of $\geq$0.0018 mg $P \cdot kg^{-1} \cdot d^{-1}$ and at an alendronate dose of 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$. Etidronate significantly reduced the extent of von Kossa staining in the abdominal aorta only at the highest dose tested, 6.25 mg $P \cdot kg^{-1} \cdot d^{-1}$. None of the bisphosphonates tested significantly reduced the hypercalcemia caused by vitamin D treatment, which remained at 40% above normal serum calcium levels at all bisphosphonate doses tested (Figure II: published online at http://atvb.ahajournals.org).

Effect of Timing of Alendronate Administration on the Inhibition of Artery Calcification

To further test the hypothesis that artery calcification is linked to bone resorption, we examined the relationship between the timing of alendronate administration and the extent to which alendronate inhibits artery calcification. This experiment is based on our previous study of the time course of artery calcification in rats treated with vitamin D plus warfarin,[3] which shows that artery calcification can be detected only histologically and by chemical analysis at 48 hours after the first vitamin D injection. Animals were given the same doses of vitamin D and warfarin and were divided into 4 treatment groups based on the timing of the 0.25 mg $P \cdot kg^{-1} \cdot d^{-1}$ dose of alendronate: One group received no alendronate; a second group received alendronate continuously for the entire 8 days of the experiment; a third group received alendronate only for the first 6 days of the experiment, which is the period before the time that artery calcification can be detected in rats treated with vitamin D plus warfarin; and the last group received alendronate only for the last 2 days of the experiment, which is the period during which artery calcification occurs in rats treated with vitamin D plus warfarin. As shown in Figure 4, quantitative analysis of the accumulation of mineral phosphate in the acid demineralization extracts of the carotid arteries revealed high levels of mineral in the carotid arteries of animals that received no alendronate, intermediate levels of mineral in the carotid arteries of rats treated with alendronate for only the last 2 days of the 8-day experiment, and control levels of mineral in the carotid arteries of rats injected daily with alendronate for the entire 8 days and rats treated with alendronate for the first 6 days only. Similar results were seen by histochemical examination of mineralization in the abdominal aorta with the von Kossa stain, with massive calcification in animals that received no alendronate, reduced calcification in animals treated with alendronate only for the last 2 days of the 8-day experiment, and no evidence of calcification in animals treated with alendronate for the entire 8 days or in animals treated with alendronate for the first 6 days only (not shown). We conclude that alendronate is an effective calcification inhibitor even when it is administered only in the 6 days before the time that artery calcification typically occurs in rats treated with vitamin D plus warfarin.

Discussion

Evidence That Alendronate and Ibandronate Inhibit Artery Calcification at Doses That Do Not Produce the Impairment of Normal Bone Mineralization Previously Seen With Etidronate

The major conclusion of the present study is that low doses of alendronate and ibandronate inhibit artery calcification without causing the impairment of normal bone mineralization previously seen with etidronate. The likely reason that low doses of the newer-generation bisphosphonates were not previously tested for their ability to inhibit artery calcification is that investigators believed that bisphosphonates inhibit artery calcification and normal bone mineralization by a common physicochemical mechanism, a mechanism that predicted that artery calcification and normal bone mineralization would be inhibited at comparably high bisphosphonate doses. This belief was based largely on the fact that etidronate and other first-generation bisphosphonates tested in these early studies did inhibit artery calcification and normal bone mineralization at comparably high doses of $\approx$5 mg $P \cdot kg^{-1} \cdot d^{-1}$ (see Introduction). To quote from a 1998 review[5]: "Unfortunately, however, when administered in doses approximating those that inhibit soft tissue calcification, bisphosphonates can impair the mineralization of normal calcified tissues such as bone and cartilage and, when given in higher amounts, also dentine, enamel, and cementum"; and "The propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates in ectopic calcification." The present studies confirm that the high doses of etidronate necessary to inhibit artery calcification do indeed cause the 2 previously reported impairments in normal bone mineralization, the increase in the width of the

TABLE 2. Comparison of the Dose Dependence of the Effects of Bisphosphonates on Artery Calcification and on Bone Resorption

	Artery Calcification		Bone Resorption	
Bisphosphonate	Dose for 50% Inhibition, mg P · kg^{-1} · d^{-1}	Relative Potency	Dose for 50% Inhibition, mg P · kg^{-1} · d^{-1}	Relative Potency
Etidronate	2	1	...	1
Alendronate	0.005	400	0.01	1000
Ibandronate	0.0002	10,000	0.001	10,000

The data for the effect of daily subcutaneous dose of bisphosphonate on vitamin D–induced artery calcification are taken from Figures 3 and I (published online at http://atvb.ahajournals.org). The data for the effect of daily subcutaneous dose of alendronate and ibandronate on bone resorption are from Figure 3 in Reference 8, and the relative antiresorption potency of bisphosphonates in the rat is from Table I in Reference 33. Note that the bone resorption studies were carried out in male rats that initially weighed 200 to 230 g, and the vitamin D–induced artery calcification studies presented here were carried out in male rats that initially weighed 200 g.

osteoid seam and the increase in the width of unmineralized growth-plate cartilage,[6] but they show for the first time that artery calcification is inhibited by far lower doses of alendronate and ibandronate. In agreement with previous reports,[8,9] these low doses of alendronate and ibandronate did not produce the kinds of impaired calcification of bone seen with etidronate. A generic physicochemical mechanism for the inhibition of artery calcification by alendronate is also in conflict with the observation that alendronate is an effective calcification inhibitor even when it is administered only in the 6 days before the time that artery calcification typically occurs in rats treated with vitamin D plus warfarin.

Evidence That Alendronate and Ibandronate Inhibit Artery Calcification at Doses That Inhibit Bone Resorption

As discussed in the Introduction, the original objective of the present investigations was to test the hypothesis that artery calcification is linked to bone resorption by determining whether the selective inhibition of bone resorption with bisphosphonates will prevent artery calcification. The evidence that alendronate and ibandronate inhibit artery calcification by inhibiting bone resorption or by acting in a remarkably similar fashion at another tissue site includes the following. (1) Relative bisphosphonate dose: The relative potency of the 3 bisphosphonates studied here as inhibitors of artery calcification are identical to the relative potency of these drugs as inhibitors of bone resorption previously reported in rats of this age. These relative potencies are ibandronate $>$ alendronate $>$ etidronate (Table 2; and Fleisch[33]). (2) Actual bisphosphonate dose: The actual daily subcutaneous doses of alendronate and ibandronate necessary to inhibit artery calcification induced by warfarin plus vitamin D are close to the daily subcutaneous doses of these drugs previously shown to inhibit bone resorption (Table 2). (3) Timing of bisphosphonate dose: Alendronate is completely effective in inhibiting artery calcification even when it is administered only in the interval before the first appearance of mineral in the artery (Figure 4). Because the inhibition of bone resorption by alendronate is known to persist for $\geq$10 days after the daily administration of the drug is discontinued in male rats of this age (Figure 6 in Antic et al[15]), this result supports the prediction that the inhibition of bone resorption with alendronate will prevent artery calcification.

Hypothesis That Artery Calcification Is Linked to Bone Resorption

There are 2 ways in which the inhibition of bone resorption with low doses of alendronate and ibandronate could influence the propensity of arteries to calcify in the rat systems studied here. One possibility is that the inhibition of bone resorption could lower the concentrations of calcium and/or phosphate in blood and thereby reduces the tendency of mineral nuclei to form and grow in the artery wall. Because we observed no significant effect of alendronate or ibandronate on serum levels of calcium or phosphate in any experiment, it seems unlikely that alendronate and ibandronate inhibit artery calcification by lowering serum calcium or phosphate levels. Another possibility is that soft-tissue calcification is promoted by crystal nuclei generated at sites of bone resorption that travel in blood and occasionally lodge in soft-tissue structures. This hypothesis is supported by the observation that under some circumstances, a complex of a calcium phosphate mineral phase and MGP is released from bone and can be detected in blood and by the observation that the release of this complex from bone is inhibited by inhibitors of bone resorption (personal observations).

Alternative Hypothesis That Artery Calcification Is Initiated by the Action of Cells in the Artery Wall

A second hypothesis to account for the effectiveness of alendronate and ibandronate as inhibitors of artery calcification is that artery calcification could be initiated by the action of vascular cells in the warfarin-induced and vitamin D–induced artery calcification rat models studied here and that bisphosphonates inhibit this activity of vascular cells. This hypothesis has the advantage of placing the site of drug action at the location of the calcification. In addition, vascular cells at calcification sites have some of the phenotypic features of both osteoblasts and osteoclasts,[19,20] and vascular smooth muscle cells have been shown to calcify in cell culture.[21] Finally, amino bisphosphonates, such as alendronate and ibandronate, have recently been shown to inhibit osteoclasts by virtue of their ability to inhibit farnesyl diphosphate

synthase,[22] an enzyme found in a wide variety of cell types. The principal problem with this hypothesis is that there is as yet no evidence that any vascular cell type is affected in vivo by the low doses of bisphosphonates that affect osteoclasts and that we have shown here to inhibit artery calcification. Because bisphosphonates achieve the selective inhibition of bone resorption by virtue of their ability to concentrate on bone surfaces under the osteoclast[23] and by their uptake by osteoclasts in the process of bone resorption,[5] it is in fact unclear how cells in the vascular wall could be equivalently exposed to the low doses of alendronate and ibandronate that affect the osteoclast.

Association Between Artery Calcification and Osteoporosis

The hypothesis that artery calcification is linked to bone resorption could provide an explanation for the well documented positive association between the severity of osteoporosis in humans and the extent of calcification in the aorta[24–29] and the carotid artery.[17,30] We speculate that the link between artery calcification and osteoporosis is increased bone resorption and that in postmenopausal women this link involves the loss of the inhibitory effect of estrogen on bone resorption. This possibility is supported by the fact that artery calcification and osteoporosis both increase dramatically in women after menopause,[31,32] whereas there is no corresponding acceleration in the rate of either process in men >50 years old. There is also an association between early-onset osteoporosis and medial artery calcification in the osteoprotegerin-deficient mouse.[4] A future test of the hypothesis that the link between artery calcification and osteoporosis in humans is increased bone resorption will be to determine whether artery calcification levels are reduced in those women currently taking bisphosphonates to retard the progression of osteoporosis.

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References

1. Price PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol*. 1998;18:1400–1407.
2. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. *Nature*. 1997;386:78–81.
3. Price PA, Faus SA, Williamson MK. Warfarin-induced artery calcification is accelerated by growth and vitamin D. *Arterioscler Thromb Vasc Biol*. 2000;20:317–327.
4. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle W, Simonset WS. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev*. 1998;12:1260–1268.
5. Fleisch H. Bisphosphonates: mechanisms of action. *Endocr Rev*. 1998; 19:80–100.
6. Schenk R, Merz WA, Muhlbauer R, Rusell RGG, Fleisch H. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl2 MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calcium Tissue Res*. 1973;11:196–214.
7. Fleisch HA. Bisphosphonates: preclinical aspects and use in osteoporosis. *Ann Med*. 1997;29:55–62.
8. Muhlbauer RC, Bauss F, Schenk R, Janner M, Bosies E, Strein K, Fleisch H. BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. *J Bone Miner Res*. 1991;6:1003–1010.
9. Schenk R, Eggle P, Fleisch H, Rosini S. Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. *Calcif Tissue Int*. 1986;38:342–349.
10. Francis MD, Russell RGG, Fleisch H. Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. *Science*. 1969;165:1264–1266.
11. Fleisch HA, Russell RGG, Bisaz S, Muhlbauer RC. The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo. *Eur J Clin Invest*. 1970; 1:12–18.
12. Rosenbaum IY, Black HE, Ferrell JF. The effects of various diphosphonates on a rat model of cardiac calciphylaxis. *Calcif Tissue Res*. 1977;23:151–159.
13. Potokar M, Schmidt-Dunker M. The inhibitory effect of new diphosphonic acids on aortic and kidney calcification in vivo. *Atherosclerosis*. 1978;30:313–320.
14. Kingma JGJ, Roy PE. Effect of ethane-1-hydroxy-1,1-diphosphonate on arterial calcinosis induced by hypervitaminosis D: a morphologic investigation. *J Exp Pathol*. 1990;71:145–153.
15. Antic VN, Fleisch H, Muhlbauer RC. Effect of bisphosphonates on the increase in bone resorption induced by a low calcium diet. *Calcif Tissue Int*. 1996;58:443–448.
16. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. *Anal Chem*. 1956;28:1756–1758.
17. Barengolts EI, Berman M, Kukreja SC, Kouznetsova T, Lin C, Chomka EV. Osteoporosis and coronary atherosclerosis in asymptomatic postmenopausal women. *Calcif Tissue Int*. 1998;62:209–213.
18. Kitagawa S, Yamaguchi Y, Kunitomo M, Imaizumi N, Fujiwara M. Impairment of endothelium-dependent relaxation in aorta from rats with arteriosclerosis induced by excess vitamin D and a high-cholesterol diet. *Jpn J Pharmacol*. 1992;59:339–347.
19. Demer LL. A skeleton in the atherosclerosis closet. *Circulation*. 1995; 92:2029–2032.
20. Shanahan CM, Cary NRB, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell–mediated vascular calcification. *Circulation*. 1999;100:2168–2176.
21. Jono S, Nishizawa Y, Shioi A, Morii H. 1,25-Dihydroxyvitamin D_3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone–related peptide. *Circulation*. 1998;98: 1302–1306.
22. Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem Biophys*. 2000;373:231–241.
23. Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E, Rodan GA. Bisphosphonate action: alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest*. 1991;88: 2095–2105.
24. Boukhris R, Becker K. Calcification of the aorta and osteoporosis: a roentgenographic study. *JAMA*. 1972;219:1307–1311.
25. Fujita T, Okamoto Y, Sakagami Y, Ota K, Ohata M. Bone changes and aortic calcification in aging inhabitants of mountain versus seacoast communities in the Kii peninsula. *J Am Geriat Soc*. 1984;32:124–128.
26. Frye MA, Melton LJ, Bryant SC, Fitzpatrick LA, Wahner HW, Schwartz RS, Riggs BL. Osteoporosis and calcification of the aorta. *Bone Miner*. 1992;19:185–194.
27. Banks LM, Lees B, Macsweeney JE, Stevenson JC. Effect of degenerative spinal and aortic calcification on bone density measurements in post-menopausal women: links between osteoporosis and cardiovascular disease? *Eur J Clin Invest*. 1994;24:813–817.
28. Jie KS, Bots ML, Vermeer C, Witteman JCM, Grobbee DE. Vitamin K status and bone mass in women with and without aortic atherosclerosis: a population based study. *Calcif Tissue Int*. 1996;59:352–356.
29. Nishizawa Y, Morii H. Osteoporosis and atherosclerosis in chronic renal failure. *Osteoporos Int*. 1997;7:S188–S192.
30. Uyama O, Yoshimoto Y, Yamamoto Y, Kawai A. Bone changes and carotid atherosclerosis in postmenopausal women. *Stroke*. 1997;28: 1730–1732.
31. Elkeles A. A comparative radiological study of calcified atheroma in males and females over 50 years of age. *Lancet*. 1957;2:714–715.
32. Melton LJ, Kan SH, Frye MA, Wahner HW, O'Fallon WM, Riggs BL. Epidemiology of vertebral fractures in women. *Am J Epidemiol*. 1989; 129:1000–1011.
33. Fleisch HA. Bisphosphonates: pharmacology and use in the treatment of tumor-induced hypercalcaemia and metastatic bone disease. *Drugs*. 1991; 42:919–944.