JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 7, 2002

Bone Origin of the Serum Complex of Calcium, Phosphate, Fetuin, and Matrix Gla Protein: Biochemical Evidence for the Cancellous Bone-Remodeling Compartment

PAUL A. PRICE, JEFFREY M. CAPUTO, and MATTHEW K. WILLIAMSON

ABSTRACT

We previously described the discovery of a fetuin–matrix Gla protein (MGP)–mineral complex in the serum of rats treated with the bone-active bisphosphonate etidronate and showed that the appearance of this complex in serum correlates with the inhibition of bone mineralization by etidronate. In this study we show that the inhibition of bone resorption by treatment with the hormone calcitonin, the cytokine osteoprotegerin, or the drug alendronate, completely inhibits the generation of the fetuin-mineral complex in response to etidronate injection. These observations can be explained best by the bone-remodeling compartment (BRC), a cancellous bone compartment in which the concentrations of calcium and phosphate are determined directly by the combined actions of the osteoclast and the osteoblast. When bone mineralization is acutely inhibited by etidronate, the BRC model predicts that the continuing action of osteoclasts will cause a sharp rise in the concentrations of calcium and phosphate in the aqueous solution of the BRC with the consequent spontaneous formation of calcium phosphate crystal nuclei in which growth then would be arrested by formation of a complex with fetuin. When the inhibition of bone resorption by calcitonin, osteoprotegerin, or alendronate is combined with the acute inhibition of bone mineralization with etidronate, the BRC model correctly predicts that there will no longer be a sharp rise in calcium and phosphate, and, therefore, there will no longer be the formation of the fetuin-mineral complex. The vascular nature of the BRC is supported by the observations that the fetuin component of the fetuin-mineral complex is derived from plasma fetuin and that the fetuin mineral complex appears in plasma within minutes of the inhibition of bone mineralization with etidronate. (J Bone Miner Res 2002;17:1171–1179)

Key words: fetuin, matrix Gla protein, cancellous bone remodeling compartment, serum mineral complex

INTRODUCTION

THE PRESENT experiments are a continuation of our investigations into the mechanisms by which proteins interact specifically with mineral in vivo to prevent the calcification of arteries and other soft tissues. In a previous study,[1] we described the discovery of a high molecular weight complex of a calcium phosphate mineral phase and the proteins fetuin and matrix Gla protein (MGP) in the blood of rats injected with the bone-active bisphosphonate etidronate. The composition of this serum mineral complex consists of ~18% mineral, 80% fetuin, and 2% MGP by weight, and the presence of the complex in serum after an 8-mg/100 g etidronate dose elevates calcium by 1.8-fold (to 4.3 mM), phosphate by 1.6-fold (to 5.6 mM), and MGP by 25-fold (to 12 μg/ml). The serum mineral complex reaches maximal levels 6 h after subcutaneous injection of etidronate, and subsequently is cleared from serum by 24 h. This complex

The authors have no conflict of interest.

Division of Biology, University of California, San Diego, La Jolla, California, USA.

of fetuin, MGP, and mineral prevents the growth, aggregation, and precipitation of the mineral component, which indicates that the previously reported calcification inhibitory activities of fetuin[2,3] and MGP[4–7] may be related to their ability to form stable complexes with nascent mineral nuclei.

In our previous study, we presented evidence that etidronate generates the serum mineral complex by inhibiting bone mineralization rather than by inhibiting bone resorption.[1] This evidence includes the following observations: (1) The appearance of the serum mineral complex and the inhibition of bone mineralization both occur within about an hour after etidronate administration.[1,8] In contrast, the inhibition of bone resorption by etidronate can only be detected 1–2 days after injection of the drug.[9] (2) There is good temporal correspondence between the transient inhibition of bone mineralization and the transient appearance of the serum mineral complex after etidronate treatments spaced 24 h apart.[1] (3) The amino bisphosphonate alendronate does not generate the serum mineral complex although the doses tested were over 1000-fold above those needed to inhibit bone resorption in rats of this age. Because these alendronate doses do not inhibit normal bone mineralization,[10] the failure of alendronate to generate the serum mineral complex is compatible with the hypothesis that the complex is generated by the acute inhibition of bone mineralization.

Although etidronate apparently generates the serum mineral complex by virtue of its ability to rapidly inhibit bone mineralization, it remains possible that bone resorption is nonetheless required for this etidronate effect. The objective of this study was to determine the effect of three inhibitors of bone resorption, the hormone calcitonin, the amino bisphosphonate alendronate, and the cytokine osteoprotegerin, on the generation of the serum mineral complex in response to etidronate administration.

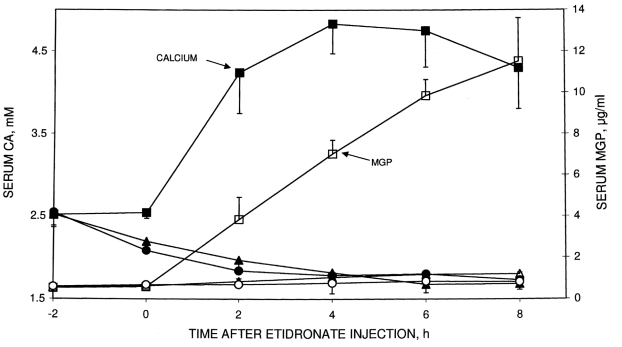

FIG. 1. Effect of calcitonin on the changes in serum levels of calcium and MGP produced by an 8 mg/100 g dose of etidronate. Twelve 40-day-old male rats were divided into three groups of four animals each. The etidronate group (calcium, ■; MGP, □) received an injection of 8 mg of etidronate/100 g body weight at $t = 0$; the calcitonin group (calcium, ▲; MGP, △) received injections of 500 ng of calcitonin/100 g body weight at $t = -2$, 0, and +2 h; and the etidronate plus calcitonin group (calcium, ●; MGP, ○) received injections of 8 mg of etidronate/100 g at $t = 0$ and of 500 ng of calcitonin/100 g at $t = -2$, 0, and +2 h. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium and MGP (see Materials and Methods section). Each data point is the average of the individually determined levels in the four experimental animals in that group, and the error bars denote the SDs.

MATERIALS AND METHODS

Materials

Simonsen albino male rats (Sprague–Dawley derived) were purchased from Simonsen Labs (Gilroy, CA, USA). Sephacryl S-300 HR gel filtration media was purchased from Pharmacia (Piscataway, NJ, USA). Ultrafree CL filtration devices (300-kDa cut-off) were purchased from Millipore Corp. (Bedford, MA, USA). Salmon calcitonin was purchased from Calbiochem (San Diego, CA, USA) and was prepared for injection in 0.1 M of sodium acetate buffer at pH 3 containing 1 mg/ml of bovine serum albumin (BSA). The osteoprotegerin used in this study was a generous gift of Amgen, Inc. (Thousand Oaks, CA, USA) and is a chimeric form of osteoprotegerin consisting of the ligand-binding domain of human osteoprotegerin (amino acids 22–194) fused at the N terminus to the C terminus of the Fc domain of human immunoglobulin G1 and is covalently dimerized through the Fc domain.[11] The calcium-deficient (diet 960177) and synthetic basal (AIN 76) diets were obtained from ICN (Aurora; Cincinnati, OH, USA). Etidronate (Didronel; Procter and Gamble, Cincinnati, OH, USA) and alendronate (Fosamax; Merck & Co., Inc., West Point, PA, USA) were purchased from University City Pharmacy (San Diego, CA, USA). Stock solutions of alendronate and etidronate were prepared for injection in 0.15 M of NaCl, titrated to pH 7.4 with NaOH and stored at 4°C. All other reagents used were reagent grade or better.

Maintenance of animals

All animal experiments were approved by the University of California, San Diego (UCSD) animal subjects committee. Unless otherwise indicated, rats were fed rodent diet 5001 (Purina Mills, Inc., St. Louis, MO, USA), a diet that is 0.67% phosphorus and 0.95% calcium by weight. In the initial calcitonin experiment (Fig. 1), 12 40-day-old male rats were divided into three groups of four animals each. The etidronate group received a single subcutaneous injection of 8 mg of etidronate/100 g body weight at $t = 0$; the calcitonin group received subcutaneous injections of 500 ng of calcitonin/100 g body weight at $t = -2$, 0, and +2 h; and the etidronate plus calcitonin group received 8 mg of etidronate/100 g body weight at $t = 0$ and 500 ng of calcitonin/100 g at $t = -2$, 0, and +2 h. At the times indicated in Fig. 1, each rat was anesthetized with metofane and a 400-μl sample of blood was withdrawn from the jugular vein. Blood samples were allowed to clot for 15 minutes at room temperature and serum was obtained after centrifugation in a clinical centrifuge and then frozen rapidly on dry ice and stored at −70°C until biochemical analysis. Analyses of the high molecular weight complex of calcium, phosphate, fetuin, and MGP (Fig. 2 and Table 1) were carried out using serum obtained at $t = 6$ h from

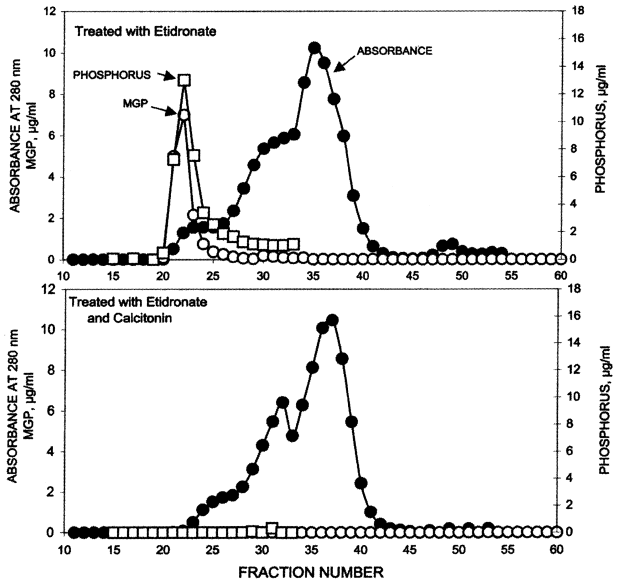

FIG. 2. Sephacryl S-300 HR filtration of serum from rats treated with etidronate alone and with etidronate plus calcitonin. Four 40-day-old male rats received a single subcutaneous dose of 8 mg of etidronate/100 g body weight at $t = 0$. Two of these animals also received subcutaneous injections of 500 ng of calcitonin/100 g at $t = -2$, 0, and +2 h. Blood was collected from each animal at $t = 6$ h and serum from the two animals in each treatment group was pooled. One-milliliter aliquots of the pooled serum samples then were applied to a 25-ml column of Sephacryl S-300 HR equilibrated with 20 mM of HEPES, pH 7.4, 0.15 M of NaCl, and 10 mM of $CaCl_2$. Temperature, 22°C; fraction size, ~0.5 ml. ●——●, Absorbance at 280 nm; ○——○, micrograms per milliliter of MGP as determined by radioimmunoassay; □——□, micrograms per milliliter of phosphorus. Top, treated with etidronate; bottom, treated with etidronate and calcitonin.

animals treated with vehicle, etidronate alone, calcitonin alone, or etidronate plus calcitonin according to the foregoing treatment procedures.

In the initial alendronate experiment (Fig. 3), 12 40-day-old male rats were divided into three groups of four animals each. The etidronate group received a single subcutaneous injection of 8 mg of etidronate/100 g body weight at $t = 0$; the alendronate group received daily subcutaneous injections of 0.13 mg of alendronate/100 g body weight beginning 48 h before the etidronate injection; and the etidronate plus alendronate group received 8 mg of etidronate/100 g body weight at $t = 0$ and daily subcutaneous injections of 0.13 mg of alendronate/100 g beginning 48 h before the etidronate injection. Serum was obtained at the times indicated in Fig. 3 and analyzed to determine the levels of calcium, phosphate, and MGP. Analyses of the high molecular weight complex of calcium, phosphate, fetuin, and MGP (Fig. 4 and Table 1) were carried out using serum obtained at $t = 6$ h from animals treated with vehicle, etidronate alone, alendronate alone, or etidronate plus alendronate according to the foregoing treatment procedures.

In the osteoprotegerin experiment (Fig. 5), 12 40-day-old male rats were divided into three groups of four animals each. The etidronate group received an injection of 8 mg of etidronate/100 g body weight at $t = 0$; the osteoprotegerin group received an injection of 0.1 mg of osteoprotegerin/100 g body weight at $t = -2$ h; and the etidronate plus osteoprotegerin group received injections of 8 mg of etidronate/100 g body weight at $t = 0$ and of 0.1 mg of osteoprotegerin/100 g body weight at $t = -2$ h. Serum was obtained at the times indicated in Fig. 5 and analyzed to determine the levels of calcium, phosphate, and MGP.

In the dietary calcium-deficiency experiment, 8 39-da-old male rats were divided into two groups of four rats each. One group was fed the calcium-deficient diet beginning 24 h before the etidronate administration, and the other group received a calcium-replete synthetic diet. On the following day, each rat was anesthetized with metofane and a 500-μl sample of blood was withdrawn from the heart. The animals then received a single subcutaneous dose of 8 mg of etidronate/100 g body weight at $t = 0$ and were exsanguinated at $t = 6$ h. Serum collected before etidronate administration and at $t = 6$ h was analyzed to determine the level of calcium, phosphate, and MGP.

Biochemical characterization of the complex between calcium, phosphate, fetuin, and MGP

For determination of MGP, aliquots of fractions and serum samples were diluted into buffer and assayed in triplicate using radioimmunoassay procedures previously described.[12] Calcium levels in serum and other samples were determined colorimetrically using cresolphthalein complexone (Sigma, St. Louis, MO, USA) and phosphate levels in serum, effluent fractions, and other samples were determined colorimetrically as described.[13] Electrophoresis was carried out using 4–20% polyacrylamide gels (Novex, Inc., San Diego, CA, USA) run in Tris-glycine buffer containing SDS.

The amount of the high molecular weight serum mineral complex in rats treated with etidronate and other agents was determined by gel filtration using 25-ml columns of Sephacryl S-300 HR equilibrated with 20 mM of HEPES, pH 7.4, 0.15 M of NaCl, and 10 mM of $CaCl_2$ at room temperature as described previously.[1] Fractions of 0.5 ml were collected and analyzed to determine the level of phosphate and MGP. The amount of the serum mineral complex in rats treated with etidronate and other agents also were determined using Ultrafree CL filtration devices with a 300-kDa molecular weight cut-off membrane. One-milliliter aliquots of serum were placed into the filter device and centrifuged for 80 minutes at 2500*g* to force the sample through the membrane. The filtrate and retentate then were analyzed to determine calcium, phosphate, MGP, and volume.

RESULTS

Effect of calcitonin on the generation of the fetuin-mineral complex after etidronate injection

The first test to determine the importance of bone resorption for the generation of the serum complex of calcium, phosphate, fetuin, and MGP was carried out using calcito-

TABLE 1. EFFECT OF BONE RESORPTION INHIBITORS ON THE GENERATION OF THE FETUIN-MINERAL COMPLEX IN ETIDRONATE-TREATED RATS

Experiment A: Calcitonin treatment

	Calcium (μmol)				*Phosphate (μmol)*				*MGP (ng)*			
	Control	*Etidronate*	*Etidronate + calcitonin*	*Calcitonin*	*Control*	*Etidronate*	*Etidronate + calcitonin*	*Calcitonin*	*Control*	*Etidronate*	*Etidronate + calcitonin*	*Calcitonin*
Initial serum	2.6	4.6	1.6	1.6	3.6	5.6	2.3	2.5	730	7090	670	500
Filtrate	0.9	0.9	0.8	0.8	2.4	3.1	1.7	1.8	20	70	70	60
Retentate	1.2	2.9	0.8	0.8	0.7	1.7	0.4	0.6	560	4160	570	390

Experiment B: Alendronate pretreatment

	Calcium (μmol)				*Phosphate (μmol)*				*MGP (ng)*			
	Control	*Etidronate*	*Etidronate + alendronate*	*Alendronate*	*Control*	*Etidronate*	*Etidronate + alendronate*	*Alendronate*	*Control*	*Etidronate*	*Etidronate + alendronate*	*Alendronate*
Initial serum	2.6	4.1	2.6	2.3	3.1	4.4	3.2	2.9	330	7040	540	230
Filtrate	1.0	1.1	1.1	0.9	2.5	2.8	2.5	2.1	50	100	50	50
Retentate	1.1	2.5	1.1	1.0	0.6	1.4	0.6	0.6	300	7030	530	190

Rats were treated with calcitonin or alendronate to inhibit bone resorption, and the effect of treatment on the presence of the fetuin-mineral complex was assessed by filtration of serum obtained 6 h after treatment with 8 mg of etidronate/100 g body weight. The data show the average micromoles of calcium and phosphate and the nanograms of MGP in 1 ml of serum before filtration through a 300-kDa molecular weight cut-off membrane and in the 0.71-ml filtrate and 0.24-ml retentate volumes recovered after filtration. There were two rats in each treatment group. See Materials and Methods section for details.

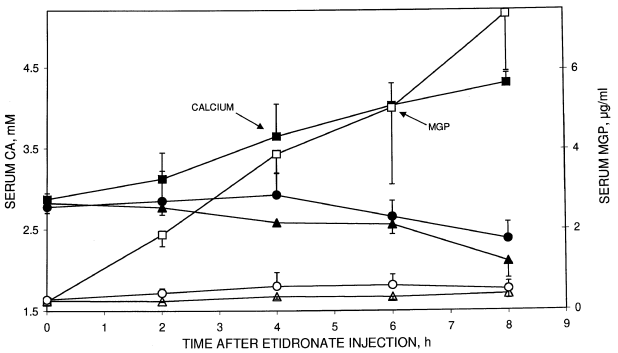

FIG. 3. Effect of alendronate pretreatment on the changes in serum levels of calcium and MGP produced by an 8-mg/100 g dose of etidronate. Twelve 40-day-old male rats were divided into three groups of four animals each. The etidronate group (calcium ■; MGP, □) received an injection of 8 mg of etidronate/100 g body weight at $t = 0$; the alendronate group (calcium, ▲; MGP, △) received injections of 0.13 mg of alendronate/100 g at $t = -48$, -24, and 0 h; and the etidronate plus alendronate group (calcium, ●; MGP, ○) received injections of 8 mg of etidronate/100 g body weight at $t = 0$ and of 0.13 mg of alendronate/100 g body weight at $t = -48$, -24, and 0 h. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium and MGP (see Materials and Methods section). Each data point is the average of the individually determined levels in the four experimental animals in that group, and the error bars denote the SDs.

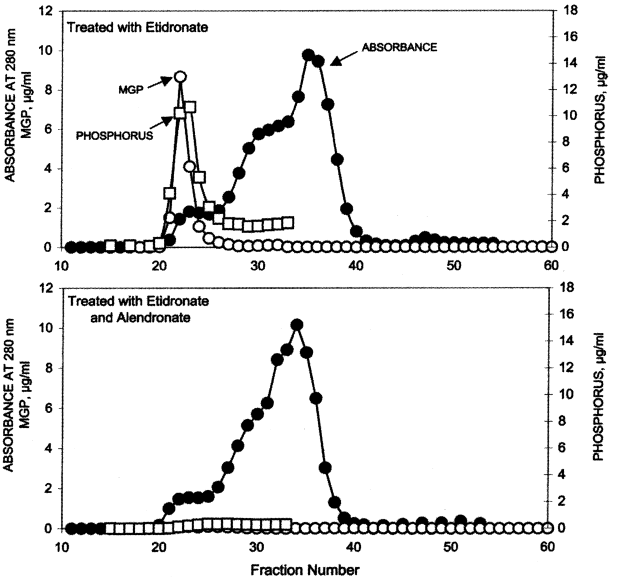

FIG. 4. Sephacryl S-300 HR filtration of serum from rats treated with etidronate alone and with etidronate plus alendronate. Four 40-day-old male rats received a single subcutaneous dose of 8 mg of etidronate/100 g body weight at $t = 0$. Two of these animals also received daily subcutaneous injections of 0.13 mg of alendronate/100 g body weight beginning 48 h before the etidronate injection. Blood was collected from each animal at $t = 6$ h and serum from the two animals in each treatment group was pooled. One-milliliter aliquots of the pooled serum samples then were applied to a 25-ml column of Sephacryl S-300 HR equilibrated with 20 mM of HEPES, pH 7.4, 0.15 M of NaCl, and 10 mM of $CaCl_2$. Temperature, 22°C; fraction size, ~0.5 ml. ●——●, Absorbance at 280 nm; ○——○, micrograms per milliliter of MGP as determined by radioimmunoassay; □——□, micrograms per milliliter of phosphorus. Top, treated with etidronate; bottom, treated with etidronate and alendronate.

nin, a hormone that potently inhibits bone resorption and produces a transient lowering of serum calcium levels. The calcitonin dose used in this test is that which produces the maximum lowering in serum calcium levels, and calcitonin was administered beginning 2 h before etidronate to ensure that the inhibition of bone resorption by the hormone was complete at the time of etidronate injection. As can be seen in Fig. 1, treatment with etidronate alone produced the same increase in serum calcium and MGP levels seen in earlier studies[1] and serum calcium and MGP levels in rats treated with etidronate plus calcitonin were nearly identical to the levels seen in rats treated with calcitonin alone. Similar results were obtained for serum phosphate levels, with the expected increase in rats treated with etidronate alone and no difference in serum phosphate between rats treated with etidronate plus calcitonin and rats treated with calcitonin alone (data not shown). This experiment has been repeated three times, and in each instance calcitonin treatment completely inhibited the increase in serum calcium, phosphate, and MGP in rats treated with etidronate.

Previous studies have shown that the increase in serum calcium, phosphate, and MGP after etidronate injection is caused by the appearance in serum of a high molecular weight complex of calcium, phosphate, MGP, and fetuin.[1] To confirm that the effects of calcitonin on total serum levels of calcium, phosphate, and MGP are indeed caused by reduced levels of this serum mineral complex, serum obtained from rats treated with etidronate alone and with etidronate plus calcitonin was subjected to gel filtration over Sephacryl S300HR and to filtration through a 300-kDa membrane. Figure 2 shows that treatment with etidronate alone produced the same high molecular weight peak of phosphate and MGP seen in previous etidronate studies[1] and treatment with etidronate plus calcitonin completely eliminated this high molecular weight component. SDS gel electrophoresis further showed the same amount of the 59-kDa fetuin component in the high molecular weight peak fractions as seen previously with the etidronate-treated rats,[1] and no significant 59-kDa component in the corresponding fractions of serum from rats treated with calcitonin plus etidronate (gel not shown). Table 1 shows that treatment with etidronate alone also produced the same increases in the high molecular weight retentate levels of calcium, phosphate, and MGP seen in previous studies[1] and treatment with calcitonin plus etidronate prevented these increases. These observations show that calcitonin treatment does in fact prevent the generation of the high molecular weight serum complex of calcium, phosphate, fetuin, and MGP after etidronate injection.

Effect of alendronate on the generation of the fetuin-mineral complex after etidronate administration

The second test to determine the importance of bone resorption for the generation of the serum complex of cal-

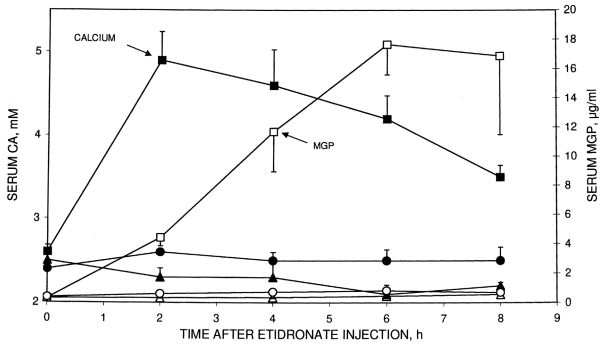

FIG. 5. Effect of osteoprotegerin on the changes in serum levels of calcium and MGP produced by an 8-mg/100 g dose of etidronate. Twelve 40-day-old male rats were divided into three groups of four animals each. The etidronate group (calcium, ■; MGP, □) received an injection of 8 mg of etidronate/100 g body weight at $t = 0$; the osteoprotegerin group (calcium, ▲; MGP, △) received an injection of 0.1 mg of osteoprotegerin/100 g at $t = -2$ h; and the etidronate plus osteoprotegerin group (calcium, ●; MGP, ○) received injections of 8 mg of etidronate/100 g body weight at $t = 0$ and of 0.1 mg of osteoprotegerin/100 g body weight at $t = -2$ h. Blood was removed from each animal at the indicated times and analyzed to determine the levels of calcium and MGP (see Materials and Methods section). Each data point is the average of the individually determined levels in the four experimental animals in that group, and the error bars denote the SDs.

cium, phosphate, fetuin, and MGP was carried out using alendronate, a bisphosphonate that potently inhibits bone resorption. The daily 0.13-mg/100 g alendronate dose used in these experiments was shown previously to inhibit bone resorption in rats of this age,[9] and alendronate was administered beginning 2 days before etidronate to ensure that bone resorption was inhibited completely at the time of etidronate injection. As can be seen in Fig. 3, treatment with etidronate alone increased serum calcium and MGP levels and serum calcium and MGP levels in rats pretreated with alendronate and then treated with etidronate were nearly identical to the levels seen in rats that did not receive etidronate. Similar results were obtained for serum phosphate levels, with an increase in rats treated with etidronate alone, and no difference in serum phosphate between rats treated with etidronate plus alendronate and rats that did not receive etidronate (data not shown). This experiment has been repeated using an alendronate pretreatment period of 3 days rather than 2 days, and alendronate again produced a comparable inhibition of the increase in serum calcium, phosphate, and MGP at 6 h after etidronate injection.

To confirm that the effects of alendronate on total serum levels of calcium and MGP are caused by reduced levels of the high molecular weight serum mineral complex, serum obtained from rats treated with etidronate alone and with etidronate plus alendronate was subjected to gel filtration over Sephacryl S300HR and filtration through a 300-kDa membrane. Figure 4 shows that alendronate pretreatment completely inhibited the appearance of the high molecular weight peak of phosphate and MGP seen in the gel filtration of serum from rats treated with etidronate alone. Table 1 shows that alendronate pretreatment also prevented the increase in the high molecular weight retentate levels of calcium, phosphate, and MGP after etidronate injection.

Effect of osteoprotegerin on the generation of the fetuin-mineral complex after etidronate administration

The third test to determine the importance of bone resorption for the generation of the serum complex of calcium, phosphate, fetuin, and MGP was carried out using osteoprotegerin, a secreted protein that potently inhibits bone resorption. The 0.1-mg/100 g body weight osteoprotegerin dose used in this experiment has been shown previously to inhibit bone resorption in rats of this age.[11,14,15] As can be seen in Fig. 5, treatment with etidronate alone produced the same increase in serum calcium and MGP levels seen previously[1] and serum calcium and MGP levels in rats treated with etidronate plus osteoprotegerin were nearly identical to the levels seen in rats treated with osteoprotegerin alone. Similar results were obtained for serum phosphate levels, with the expected increase in rats treated with etidronate alone, and no significant difference in serum phosphate between rats treated with etidronate plus osteoprotegerin and rats treated with osteoprotegerin alone (data not shown).

Effect of feeding a calcium-deficient diet for 1 day on the generation of the fetuin-mineral complex after etidronate administration

To further confirm the bone origin of the serum mineral complex, additional experiments were carried out to evaluate intestinal calcium adsorption as a potential source of the calcium found in the serum mineral complex. Rats were first placed onto a synthetic calcium-deficient diet 1 day before etidronate administration to remove calcium from the intestine, and the levels of calcium, phosphate, and MGP then were measured in serum obtained 6 h after etidronate injection. As seen in Table 2, the increases in serum calcium, phosphate, and MGP in the animals fed the calcium-deficient diet for 1 day were comparable with the increases found in animals fed a calcium-replete synthetic diet for 1 day. Although the levels of serum calcium and phosphate at $t = 6$ h were slightly higher for the animals fed the calcium-deficient diet in this experiment, this difference was not significant for serum phosphate ($p > 0.5$) and only barely significant for serum calcium ($0.025 < p < 0.05$). Because the opposite trend was observed in a repeat experiment, with slightly higher levels of serum calcium and phosphate at $t =$ 6 h in the animals fed the calcium-replete synthetic diet for 1 day, we conclude that there probably is no significant effect of feeding a calcium-deficient diet for 1 day on the serum mineral response to etidronate. These results show that the calcium component of the mineral complex found in the serum from etidronate-treated rats cannot arise directly from intestinal calcium adsorption and, therefore, further support bone metabolism as the probable origin of the serum mineral complex.

TABLE 2. EFFECT OF FEEDING A CALCIUM-DEFICIENT DIET FOR 1 DAY ON THE GENERATION OF THE SERUM MINERAL COMPLEX IN ETIDRONATE-TREATED RATS

	Calcium (μmol)		*Phosphate (μmol)*		*MGP (ng)*	
Diet type	*0 h*	*6 h*	*0 h*	*6 h*	*0 h*	*6 h*
Calcium replete	2.8 ± 0.1	4.4 ± 0.6	3.0 ± 0.2	5.2 ± 0.5	333 ± 37	7465 ± 1630
Calcium deficient	2.8 ± 0.1	5.4 ± 0.5	3.0 ± 0.3	6.1 ± 0.3	267 ± 31	8083 ± 165

Four rats were placed on a calcium-deficient diet and four rats were placed on a calcium-replete synthetic diet on arrival. The next morning, all rats were injected with 8 mg of etidronate/100 g body weight. Blood was obtained immediately before injection of etidronate ($t = 0$) and at $t = 6$ h. Serum calcium, phosphorus, and MGP were determined as described in the Materials and Methods section. The data shown are the mean ± SD values for 1 ml of serum for the four rats in each treatment group.

DISCUSSION

This study shows that bone resorption activity is required for the generation of the complex of fetuin, MGP, and mineral previously discovered in the blood of rats after injection with high doses of etidronate. The first experiments show that doses of the hormone calcitonin that inhibit bone resorption can prevent the increase in total serum levels of calcium, phosphate, and MGP after etidronate administration. Calcitonin also prevents the appearance of the high molecular weight complex of fetuin, MGP, calcium, and phosphate, which is responsible for the increases in these serum parameters in the etidronate-treated rat.[1] The second experiments show that pretreatment with the amino bisphosphonate alendronate using doses known to inhibit bone resorption[9] also inhibits the increase in total serum levels of calcium, phosphate, and MGP after etidronate administration as well as the appearance of the high molecular weight complex of fetuin, MGP, calcium, and phosphate, which is responsible for the increases in these serum parameters. The third experiments show that doses of the cytokine osteoprotegerin known to inhibit bone resorption inhibit the increase in total serum levels of calcium, phosphate, and MGP after etidronate administration. Taken together, these observations provide convincing evidence that osteoclastic bone resorption indeed is required for the formation of the fetuin-mineral complex in response to etidronate injection.

In a previous study[1] we presented evidence that etidronate actually generates the fetuin-mineral complex by inhibiting bone mineralization rather than by inhibiting bone resorption (see the Introduction section). If etidronate indeed generates the fetuin-mineral complex by inhibiting bone mineralization, why does the inhibition of bone resorption prevent the generation of the fetuin-mineral complex? The explanation for this seeming paradox may lay in the structure of the bone-remodeling compartment (BRC), a recently described anatomical structure that is found on the surfaces of bone adjacent to bone marrow and is associated intimately with cancellous bone remodeling.[16] The BRC is lined on its marrow side by flattened cells that are in continuity with the bone-lining cells at the margins of the compartment. The remodeling bone surface forms the osseous side of the BRC, with osteoclasts actively engaged in bone resorption and osteoblasts actively engaged in bone formation. The BRC is the cancellous bone form of the bone multicellular unit, a temporary structure in which bone remodeling is accomplished by the actions of osteoclasts and osteoblasts. The more familiar cortical bone form of the bone multicellular unit is found in human bone but not in the bone of normal rats.[17]

The BRC structure defines an aqueous phase in which the concentration of calcium and phosphate are determined by the combined actions of the osteoclast and the osteoblast. When bone mineralization is acutely inhibited by etidronate, the BRC structure predicts that the continuing action of osteoclasts will cause a sharp rise in the concentrations of calcium and phosphate in the aqueous solution of the BRC. We speculate that this leads to the spontaneous formation of calcium phosphate crystal nuclei within the BRC in which growth then is arrested by formation of a specific complex with fetuin and MGP. This possibility is supported by the observation that a similar fetuin-mineral complex indeed forms when calcium and phosphate are added to rat serum or plasma in vitro (P. A. Price, unpublished data, 2001). When the inhibition of bone resorption by agents such as calcitonin is combined with the acute inhibition of bone mineralization with etidronate, the BRC structure correctly predicts that there will no longer be a sharp rise in calcium and phosphate and, therefore, there will no longer be the formation of the fetuin-mineral complex.

There is evidence that the BRC also is a vascular compartment,[18] evidence that includes the presence of numerous red blood cells within the compartment and the close association of the compartment with blood vessels. This vascular feature of the BRC could provide an explanation for two experimental observations: (1) the observation that plasma fetuin is consumed in the course of formation of the fetuin-mineral complex,[1] and (2) the observation that the fetuin-mineral complex appears rapidly in blood plasma, attaining plasma levels that are already 25% of maximum in just 30 minutes after etidronate injection.[1] It would be difficult to account for either observation unless the BRC is indeed a vascular compartment.

There may be other circumstances in which an excess of bone resorption over bone formation leads to the formation of calcium phosphate crystal nuclei that escape from the BRC and appear in blood, although this remains to be established. Once in blood, it is further possible that some of these crystal nuclei occasionally lodge in the elastic lamel-

lae of arteries and in other extracellular structures in soft tissues, where they may initiate calcification. We believe that this process of seeded crystal growth is the dominant mechanism for the initiation of artery calcification, and cite in support of this hypothesis the observation that even normal regions of the human artery media contain innumerable calcium phosphate crystallites[19] that are coated with MGP molecules.[20] The results of several previous studies are consistent with this model: (1) The postmenopausal women with the most rapid bone loss (and therefore the greatest imbalance between resorption and formation within the bone remodeling compartment) have the most rapid rate of artery calcification.[21–27] (2) Mice in which bone resorption is accelerated by the targeted deletion of the osteoprotegerin gene have early onset osteoporosis (and an imbalance between resorption and formation within the BRC) as well as focal calcification of the artery media.[28,29] (3) The amino bisphosphonates alendronate and ibandronate and the cytokine osteoprotegerin all inhibit warfarin-induced artery calcification in the rat at doses that are comparable with the doses that inhibit bone resorption.[30,31] These observations strongly support the present model for artery calcification, because inhibition of bone resorption would be expected to eliminate conditions in which resorption exceeds formation within the BRC and thereby prevent the formation of the supersaturated concentrations of calcium and phosphate within the solution of the BRC that lead to formation of crystal nuclei.

ACKNOWLEDGEMENTS

The authors acknowledge helpful discussions with Ellen Hauge on the structure and function of the BRC. This work was supported in part by U.S. Public Health Service grant AR25921.

REFERENCES

1. Price PA, Thomas GT, Pardini AW, Figueira WF, Caputo J, Williamson MK 2002 Discovery of a high molecular weight complex of calcium, phosphate, fetuin, and matrix Gla protein in the serum of etidronate-treated rats. J Biol Chem **277:**3926–3934.
2. Schinke T, Amendt C, Trindl A, Poschke O, Muller-Esterl W, Jahnen-Dechent W 1996 The serum protein alpha 2-HS Glycoprotein/Fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. J Biol Chem **271:**20789–20796.
3. Jahnen-Dechent W, Schinke T, Trindl A, Muller-Esterl W, Sablitzky F, Kaiser S, Blessing M 1997 Cloning and targeted deletion of the mouse fetuin gene. J Biol Chem **272:**31496–31503.
4. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G 1997 Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. Nature **386:**78–81.
5. Price PA, Faus SA, Williamson MK 1998 Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. Arterioscler Thromb Vasc Biol **18:**1400–1407.
6. Munroe PB, Olgunturk RO, Fryns JP, Maldergem LV, Ziereisen F, Yuksel B, Gardiner RM, Chung E 1999 Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. Nat Genet **21:**142–144.
7. Price PA, Faus SA, Williamson MK 2000 Warfarin induced artery calcification is accelerated by growth and by vitamin D. Arterioscler Thromb Vasc Biol **20:**317–327.
8. Schenk R, Merz WA, Muhlbauer R, Rusell RGG, Fleisch H 1973 Effect of Ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. Calcif Tissue Res **11:**196–214.
9. Antic VN, Fleisch H, Muhlbauer RC 1996 Effect of bisphosphonates on the increase in bone resorption induced by a low calcium diet. Calcif Tissue Int **58:**443–448.
10. Muhlbauer RC, Bauss F, Schenk R, Janner M, Bosies E, Strein K, Fleisch H 1991 BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. J Bone Miner Res **6:**1003–1010.
11. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Amgen EST Program, Boyle WJ 1997 Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell **89:**309–319.
12. Otawara Y, Price PA 1986 Developmental appearance of matrix Gla protein during calcification in the rat. J Biol Chem **261:**10828–10832.
13. Chen PS, Toribara TY, Warner H 1956 Microdetermination of phosphorus. Anal Chem **28:**1756–1758.
14. Morony S, Capparelli C, Lee R, Shimamoto G, Boone T, Lacey DL, Dunstan CR 1999 A chimeric form of osteoprotegerin inhibits hypercalcemia and bone resorption induced by IL-1β, TNF-a, PTH, PTHrP, and 1,25$(OH)_2D_3$. J Bone Miner Res **14:**1478–1485.
15. Bateman TA, Dunstan CR, Ferguson VL, Lacey DL, Ayers RA, Simske SJ 2000 Osteoprotegerin mitigates tail suspension-induced osteopenia. Bone **26:**443–449.
16. Hauge EM, Qvesel D, Eriksen EF, Mosekilde L, Melsen F 2001 Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. J Bone Miner Res **16:**1575–1582.
17. Frost HM, Jee WSS 1992 On the rat model of human osteopenias and osteoporoses. Bone Miner **18:**227–236.
18. Parfitt AM 2001 The bone remodeling compartment: A circulatory function for bone lining cells. J Bone Miner Res **16:**1583–1585.
19. Reid JD, Andersen ME 1993 Medial calcification (whitlockite) in the aorta. Atherosclerosis **101:**213–224.
20. Alagao FC, Patel R, Price PA 2000 Matrix Gla protein specifically inhibits calcification of human aortic elastin in vitro. J Bone Miner Res **15:**S1;S208.
21. Barengolts EI, Berman M, Kukreja SC, Kouznetsova T, Lin C, Chomka EV 1998 Osteoporosis and coronary atherosclerosis in asymptomatic postmenopausal women. Calcif Tissue Int **62:**209–213.
22. Boukhris R, Becker K 1972 Calcification of the aorta and osteoporosis: A roentgenographic study. J Am Med Assoc **219:**1307–1311.
23. Banks LM, Lees B, Macsweeney JE, Stevenson JC 1994 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 **24:**813–817.
24. Frye MA, Melton LJ, Bryant SC, Fitzpatrick LA, Wahner HW, Schwartz RS, Riggs BL 1992 Osteroporosis and calcification of the aorta. Bone Miner **19:**185–194.
25. Jie KS, Bots ML, Vermeer C, Witteman JCM, Grobbee DE 1996 Vitamin K status and bone mass in women with and without aortic atherosclerosis: A population based study. Calcif Tissue Int **59:**352–356.

26. Nishizawa Y, Morii H 1997 Osteoporosis and atherosclerosis in chronic renal failure. Osteoporos Int **7:**S188–S192.
27. Uyama O, Yoshimoto Y, Yamamoto Y, Kawai A 1997 Bone changes and carotid atherosclerosis in postmenopausal women. Stroke **28:**1730–1732.
28. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle W, Simonset WS 1998 Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev **12:**1260–1268.
29. Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, Sato Y, Nakagawa N, Yasuda H, Mochizuki S, Gomibuchi T, Yano K, Shima N, Washida N, Tsuda E, Morinaga T, Higashio K, Ozawa H 1998 Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun **247:**610–615.
30. Price PA, Faus SA, Williamson MK 2001 The bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those which inhibit bone resorption. Arterioscler Thromb Vasc Biol **21:**817–824.
31. Price PA, June HH, Buckley JR, Williamson MK 2001 Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. Arterioscler Thromb Vasc Biol **21:**1610–1616.

Address reprint requests to:
Paul A. Price, Ph.D.
Division of Biology, 0368
University of California, San Diego
La Jolla, CA 92093-0368, USA

Received in original form December 3, 2001; in revised form January 25, 2002; accepted February 28, 2002.