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Serum Levels of the Fetuin-Mineral Complex Correlate with Artery Calcification in the Rat*[S]

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Paul A. Price‡, Matthew K. Williamson, Thao Minh Thi Nguyen, and Truclinh N. Than

From the Division of Biology, University of California, La Jolla, California 92093-0368

The present studies were carried out to evaluate the possible association between the presence of the fetuin-mineral complex in serum and vitamin D-induced artery calcification. The first experiment shows that there is a fetuin-mineral complex in the blood of rats in which extensive calcification of the artery media has been induced by treatment with vitamin D for 96 h, and that there is no detectable fetuin-mineral complex in the blood of rats in which artery calcification has been inhibited by concurrent treatment with ibandronate or osteoprotegerin. The second experiment shows that the timing of vitamin D-induced artery calcification correlates with the timing of the maximal increase in serum fetuin-mineral complex levels. Whereas both results indicate that serum levels of the fetuin-mineral complex are indeed associated with vitamin D-induced artery calcification, the biochemical basis for this association is presently unclear. One possibility is that high levels of the fetuin-mineral complex cause defects in the ability of fetuin to prevent the growth of the mineral component, which then seeds artery calcification. Another possibility is that the fetuin-mineral complex is the downstream product of a pathway that begins with the true causative agent, and that the serum level of the fetuin-mineral complex is a marker for the activity of this agent in blood. An unexpected finding of the present studies is that vitamin D-induced artery calcification is also correlated with a 65 to 75% reduction in serum fetuin, a reduction that appears to be caused by the clearance of the fetuin-mineral complex from serum.

We recently proposed the hypothesis that artery calcification is linked to bone resorption to explain the association between increased bone resorption and increased artery calcification that has been seen in the vitamin D-treated rat (1), in the osteoprotegerin-deficient mouse (2), and in patients with postmenopausal osteoporosis (see Ref. 3 for references). One prediction of the hypothesis that artery calcification is linked to bone resorption is that inhibitors of bone resorption should inhibit artery calcification (3). In previous studies we tested this prediction using three different types of bone resorption inhibitors, each with an entirely different mode of action on the osteoclast: the amino bisphosphonates alendronate and ibandronate (3), the cytokine osteoprotegerin (4), and the V-H^+-ATPase inhibitor SB 242784 (5). Each bone resorption inhibitor potently inhibited artery calcification.

Our working hypothesis is that a causative agent for artery calcification arises in bone, travels in blood, and then induces calcification in the artery wall. A necessary condition for this blood-born theory of artery calcification is that bone resorption inhibitors affect blood chemistry in a way that is plausibly associated with their ability to prevent artery calcification. In previous studies we have shown that this putative change in blood chemistry cannot simply be changes in serum calcium and phosphate, because doses of osteoprotegerin and of bisphosphonates that inhibit artery calcification do not lower serum levels of calcium or phosphate in the warfarin-treated rat, and do not affect the extent of hypercalcemia seen in the vitamin D-treated rat (3, 4, 6).

In the present investigations we have examined another potential change in blood chemistry that could be arguably correlated with artery calcification, the presence of a fetuin-mineral complex. Previous studies have shown that the fetuin-mineral complex consists of a calcium phosphate mineral phase and the proteins fetuin (called α_2-HS glycoprotein in humans) and matrix Gla protein (MGP),[1] and that the complex can be detected at extraordinarily high levels in blood following the acute inhibition of mineralization with etidronate (7). Interestingly, the inhibition of bone resorption with calcitonin, alendronate, or osteoprotegerin completely prevents the appearance of the fetuin-mineral complex in blood following etidronate administration (8). More recent studies found that the fetuin-mineral complex is formed from the serum fetuin pool, and that clearance of the complex from blood removes fetuin associated with the complex and thereby reduces total levels of serum fetuin (9). These studies also showed that the ratio of the dominant protein component of the complex, fetuin, to calcium phosphate mineral varies by as much as 1.9-fold depending on etidronate dose, and the term "fetuin-mineral complex" therefore refers to a range of complexes of fetuin, MGP, and mineral that differ somewhat in composition much like the low density lipoprotein of blood (9). In the studies presented here our general strategy has been to directly compare blood levels of the fetuin-mineral complex in rats in which artery calcification had been induced by treatment with toxic doses of vitamin D to those found in rats treated with toxic doses of vitamin D together with doses of ibandronate or osteoprotegerin sufficient to completely prevent any evidence of vitamin D-induced artery calcification.

The dominant protein component of the fetuin-mineral complex, fetuin, is a 59-kDa glycoprotein that consists of 2 N-terminal cystatin domains and a smaller C-terminal domain.

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. A and B.

‡ To whom correspondence should be addressed: Dept. of Biology, 0368, University of California, La Jolla, CA 92093-0368. Tel.: 858-534-2120; Fax: 858-534-1492; E-mail: pprice@ucsd.edu.

[1] The abbreviations used are: MGP, matrix Gla protein; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Fetuin is synthesized in the liver and is found at high concentrations in mammalian serum (10, 11) and bone (12–17). The serum fetuin concentration in adult mammals ranges from 0.5 to 1.5 mg/ml, whereas the serum fetuin concentration in the fetus and neonate is typically far higher (11). Fetuin is also one of the most abundant non-collagenous proteins found in bone (12–17), with a concentration of about 1 mg of fetuin/g of bone in rat (16), bovine (12), and human (14) bone. Despite the abundance of fetuin in bone, however, it has not been possible to demonstrate the synthesis of fetuin in calcified tissues, and it is therefore presently thought that the fetuin found in bone arises from hepatic synthesis via serum (15, 17). This view is supported by the observation that fetuin binds strongly to apatite, the mineral phase of bone, and is concentrated from serum onto apatite to a far greater extent than is albumin (13). The less abundant protein component of the complex, MGP, is a 10-kDa vitamin K-dependent protein that is secreted by a wide variety of cell types, including vascular smooth muscle cells.

Both protein components of the fetuin-mineral complex have been shown to function as calcification inhibitors. *In vitro* studies demonstrate that MGP potently inhibits calcification of aortic elastin *in vitro* (18), and genetic and biochemical studies have convincingly demonstrated that impaired MGP function causes extensive calcification of the elastic lamellae in the artery media of mice (19), rats (20), and humans (21). *In vitro* studies have also demonstrated that fetuin is an important inhibitor of apatite formation in serum containing increased levels of calcium and phosphate (22), and that targeted deletion of the fetuin gene reduces the ability of serum to arrest apatite formation by over 70% (23). More recent studies have shown that a fetuin-mineral complex is formed in the course of the fetuin-mediated arrest of apatite formation in serum containing increased calcium and phosphate (24). Purified bovine fetuin has also been shown to be a potent inhibitor of the precipitation of a calcium phosphate mineral phase from supersaturated solutions of calcium phosphate (22), and recent studies have shown that the fetuin-mineral complex is formed in the course of this inhibition (24). Despite the evidence that fetuin is a potent serum inhibitor of calcium phosphate precipitation, the phenotype of the fetuin-deficient mouse on a normal diet is far less dramatic than that of the MGP-deficient mouse, with evidence of ectopic microcalcifications in soft tissues but no evidence for artery calcification (23).

EXPERIMENTAL PROCEDURES

Materials—Vitamin D_3 (cholecalciferol) was purchased from Sigma and Ibandronate (Bondronat, Roche Diagnostics) was purchased from Idis World Medicines (Surrey, United Kingdom). Ibandronate was diluted with 0.15 M NaCl and stored at 4 °C. The osteoprotegerin used in this study was a generous gift of Amgen, Inc., 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 G_1, and is covalently dimerized through the Fc domain (25). Stock solutions of vitamin D were prepared fresh daily for each 3-day subcutaneous injection cycle at a concentration of 1.65 mg/ml in 7% Emulphor (alkamuls EL-620, Rodia, Inc.) (1). NuPAGE bis-Tris 4–12% acrylamide gels were purchased from Invitrogen Corp. (Carlsbad, CA). Simonsen albino rats (Sprague-Dawley derived) were purchased from Simonsen Labs (Gilroy, CA).

Methods—Calcium levels in serum and acid extracts were determined colorimetrically using cresolphthalein complexone (Sigma) and phosphate levels in serum and in acid extracts were determined colorimetrically as described (26). For measurement of mineral accumulation in thoracic aorta, aortas were removed within 30 min of death, cleaned of adhering non-vascular tissue, and patted dry with a kimwipe. The aorta was placed into a 2-ml polypropylene centrifuge tube, 1 ml of 150 mM HCl was added to each tube, and the tubes were mixed end over end for 24 h at room temperature to dissolve mineral. Serum samples were analyzed to determine the level of cross-linked N-telopeptides (Osteomark NTx) by Ostex, Inc. (Seattle, WA) using a rat-specific[2] enzyme-linked immunosorbent assay (27), and to determine the level of rat albumin by the University of California, San Diego, Veterinary Diagnostic Laboratory using a VetTest analyzer. For determination of rat MGP and rat fetuin, aliquots of acid extracts and serum samples were assayed in triplicate using radioimmunoassay procedures previously described (9, 28). Alizarin red staining was carried out as described (29, 30).

For measurement of serum levels of the fetuin-mineral complex, blood was allowed to clot at room temperature for 1 h, serum was collected by centrifuging at 1,400 × *g* for 10 min, and 200-μl aliquots were immediately frozen on dry ice and stored at −70 °C. The serum fetuin-mineral complex was sedimented by centrifuging the 0.2-ml aliquots of serum for 2 h at 16,000 × *g*. Each tube was rinsed briefly with 200 μl of ice-cold 0.15 M NaCl and the tubes were then incubated with 35 μl of 150 mM HCl for 24 h at room temperature. The acid extracts were analyzed for calcium, phosphate, fetuin, and MGP. In a repeat centrifugation experiment (Fig. 3), pellets were dissolved in 40 μl of SDS gel loading buffer containing 60 mM EDTA, pH 7.5, and reducing agent, and 20-μl aliquots were electrophoresed using 4–12% polyacrylamide gels.

Treatment of Animals—Rats were fed *ad libitum* with rodent diet 5001 (Purina Mills Inc., St. Louis, MO), a diet that contains 0.67% phosphorus and 0.95% calcium by weight. Animals were killed by exsanguination while under isoflurane anesthetic. Artery calcification was induced by treatment with toxic doses of vitamin D, as described previously (1). In brief, 7-week-old male rats received subcutaneous injections of 500,000 IU of vitamin D_3/kg body weight at $t = 0$, 24, and 48 h. Rats were killed 96 h after the first vitamin D injection, and the appropriate tissues were removed for analysis. In the osteoprotegerin experiment, rats also received daily subcutaneous injections of osteoprotegerin at a dose of 1 mg/kg/day beginning at the time of the first vitamin D injection. In the ibandronate experiment, rats also received subcutaneous injections of ibandronate at a dose of 0.25 mg/kg/day beginning 4 days prior to the first vitamin D injection.

In the time course study, six 7-week-old male rats served as untreated controls ($t = 0$); 6 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$ and were killed at $t = 24$ h; 6 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$ and 24 h and were killed at $t = 48$ h; and 18 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$, 24, and 48 h, 6 of these were killed at $t = 72$, 6 at $t = 84$, and 6 at $t = 96$ h. The abdominal aorta and attached arteries were dissected as a unit from each animal. Each abdominal aorta unit was first stained with Alizarin red S and then extracted with 150 mM HCl to dissolve any calcification that might be present. The UCSD Animal Subjects Committee approved all animal experiments.

RESULTS

Correlation between Artery Calcification and Serum Levels of the Fetuin-Mineral Complex in Rats Treated for 96 h with Vitamin D, Vitamin D + Ibandronate, and Vitamin D + Osteoprotegerin—Rats were treated for 3 days with 500,000 IU of vitamin D/kg, a procedure previously shown to induce extensive artery calcification (4, 6). Subsets of these animals were also treated with doses of osteoprotegerin or ibandronate known to inhibit bone resorption in rats of this age and which, in prior studies, have been shown to inhibit vitamin D-induced artery calcification (4, 6). In agreement with these earlier studies, the abdominal arteries from all 6 rats treated with vitamin D alone had extensive Alizarin red staining for calcification, whereas the arteries of the 6 rats treated with vitamin D plus ibandronate and the 6 rats treated with vitamin D plus osteoprotegerin had no detectable Alizarin red staining for calcification. Fig. A (see Supplemental Materials) shows a typical example of the level of Alizarin red staining for calcification seen in the abdominal arteries of rats treated with vitamin D alone, and examples of the absence of Alizarin red staining seen in the arteries of rats treated with vitamin D plus osteoprotegerin and in rats treated with vitamin D plus ibandro-

[2] The rat-specific NTx assay used in these tests is no longer offered by Osteomark.

nate. Note that calcification in the vitamin D-treated animals is particularly pronounced in the smaller branch arteries, such as the mesenteric, celiac, and renal arteries. In previous studies, microscopic examination of von Kossa-stained sections showed that vitamin D-induced artery calcification begins within the elastic lamellae and is absent in artery sections from rats treated with vitamin D plus osteoprotegerin and with vitamin D plus ibandronate (4, 6).

The possible presence of the fetuin-mineral complex in the serum of vitamin D-treated rats was evaluated by biochemical analysis of the pellets formed by centrifuging serum for 2 h at 16,000 $\times$ *g*, a procedure that has been previously shown to pellet the fetuin-mineral complex found in the serum of etidronate-treated rats (7). A translucent, compact pellet was found in each of the 6 serum samples from animals treated with vitamin D alone,[3] but could not be detected in any of the serum from untreated control animals or from animals treated with vitamin D plus either ibandronate or osteoprotegerin. This pellet was visually identical to the translucent fetuin-mineral complex pellet formed by centrifuging serum from etidronate-treated rats. After removal of the supernatants, 0.15 M HCl was added to each tube to dissolve any fetuin-mineral complex that might be present, and the resulting acid extracts (termed "pellet fractions") were analyzed to determine the levels of the known constituents of the serum fetuin-mineral complex, calcium, phosphate, fetuin, and matrix Gla protein (7). As seen in Fig. 1, treatment with vitamin D alone elevated the levels of calcium, phosphate, fetuin, and MGP in the acid extracts by 25–75-fold compared with the levels in the acid extracts of control serum ($p < 0.001$ compared with control values). As an additional control for this experiment, serum samples from control and vitamin D-treated rats were placed in identical tubes but not centrifuged, and the serum was then removed, 0.15 M HCl was added to each tube, and the acid extracts were analyzed for calcium, phosphate, fetuin, and MGP. The resulting values were not significantly different from the values shown in Fig. 1 for control serum after centrifugation (data not shown). These results show that there is not sufficient fetuin-mineral complex in the serum of control rats to be detected by these centrifugation procedures, whereas there is abundant centrifugation evidence for the presence of a fetuin-mineral complex in the serum of vitamin D-treated rats. Based on the pellet analyses, the weight ratio of mineral to fetuin in the fetuin-mineral complex found in the serum of vitamin D-treated rats is 1.32, which is comparable with the 1.15 ratio found for the complex in the serum of etidronate-treated rats when the same radioimmunoassay is used for fetuin measurement (9).[4]

Fig. 1 also shows that treatment with doses of ibandronate that prevent vitamin D-induced artery calcification eliminated the characteristic chemical elements of the fetuin-mineral complex from the pellet fraction. Treatment with vitamin D plus osteoprotegerin dramatically lowered the levels of these parameters compared with the values seen in the pellet fractions of animals treated with vitamin D alone, but in each case the values found in the rats treated with vitamin D plus osteoprotegerin remained slightly above those seen in untreated control animals.

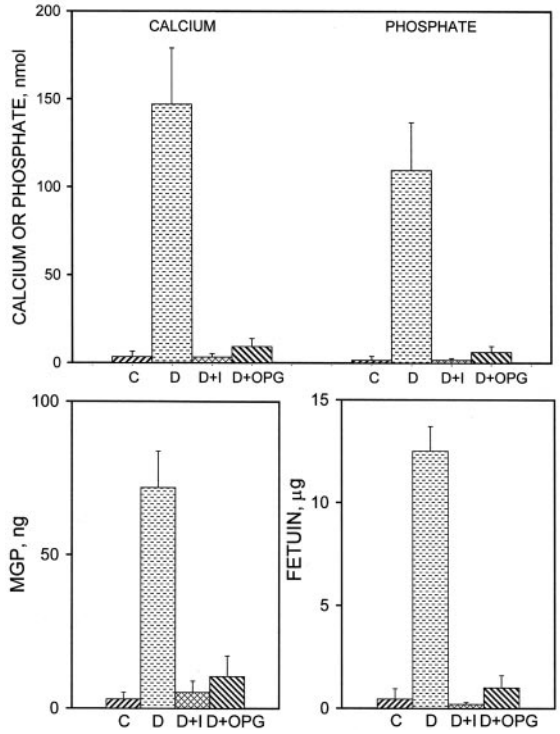

FIG. 1. **Chemical analysis of the pellet fraction obtained by centrifugation of serum from rats treated with vitamin D alone, with vitamin D plus ibandronate, and with vitamin D plus osteoprotegerin.** Eighteen 7-week-old male rats were treated for 3 days with 500,000 IU of vitamin D/kg/day to induce artery calcification. To inhibit the vitamin D-induced calcification of arteries, 6 rats also received osteoprotegerin at a dose of 1 mg/kg/day, and 6 received ibandronate at a dose of 0.25 mg/kg/day (see "Experimental Procedures"). Six additional animals were untreated controls. Animals were killed by exsanguination at 96 h after the first vitamin D injection, and 200-μl aliquots of serum from each rat were centrifuged for 2 h at 16,000 $\times$ *g* to sediment any fetuin-mineral complex that might be present. The supernatant was removed, 0.15 M HCl was added to each tube to dissolve the fetuin-mineral complex, and the acid extracts were analyzed for calcium, phosphate, fetuin, and MGP (see "Experimental Procedures"). The figure shows the mean $\pm$ S.D. for the six rats in each treatment group. *C*, age-matched control rats; *D*, rats treated with vitamin D alone; *D + I*, rats treated with vitamin D plus ibandronate; *D + OPG*, rats treated with vitamin D plus osteoprotegerin.

The protein composition of the putative fetuin-mineral complex was also examined by SDS-gel electrophoresis. Each serum sample was again centrifuged for 2 h at 16,000 $\times$ *g* to sediment the fetuin-mineral complex. The supernatants were then removed and SDS gel loading buffer containing 60 mM EDTA was added to each tube to dissolve any fetuin-mineral complex that might be present. Fig. 2 shows the SDS-gel electrophoresis of the resulting pellet fractions obtained by centrifugation of serum from 6 rats treated with vitamin D alone, from 6 rats treated with vitamin D plus ibandronate or with vitamin D plus osteoprotegerin, and from 6 untreated control rats. The electrophoretic pattern of the pellet fraction from the 6 rats treated with vitamin D alone is similar to that previously reported for the fetuin-mineral complex pellet obtained by centrifugation of serum of etidronate-treated rats (7), with a major band at 59 kDa and a minor band at 66 kDa. N-terminal protein sequencing showed that the 59-kDa band is rat fetuin and the 66-kDa band is rat serum albumin, the same protein identifications obtained for the corresponding bands for etidronate-treated rats (7). The other protein bands seen in the

[3] Previous efforts failed to detect the fetuin-mineral complex in serum from rats treated with vitamin D alone (7). The fact that the complex could be detected in the present experiments is because of the use of a longer centrifugation time (2 h *versus* 30 min) to form a more compact pellet, and to modifications in procedure that eliminated loss of the pellet during the 0.15 M NaCl wash step.

[4] The ratio of mineral to fetuin found for rats treated with the 32 mg of etidronate dose using the radioimmunoassay for rat fetuin, 1.15 (9), is significantly higher than the 0.53 ratio reported in the initial study of the complex (7). The earlier study relied on a qualitative comparison of staining intensity in SDS gels between the rat fetuin-mineral complex and purified bovine fetuin (7), and was less accurate than the immunoassay procedures used in the subsequent study (9).

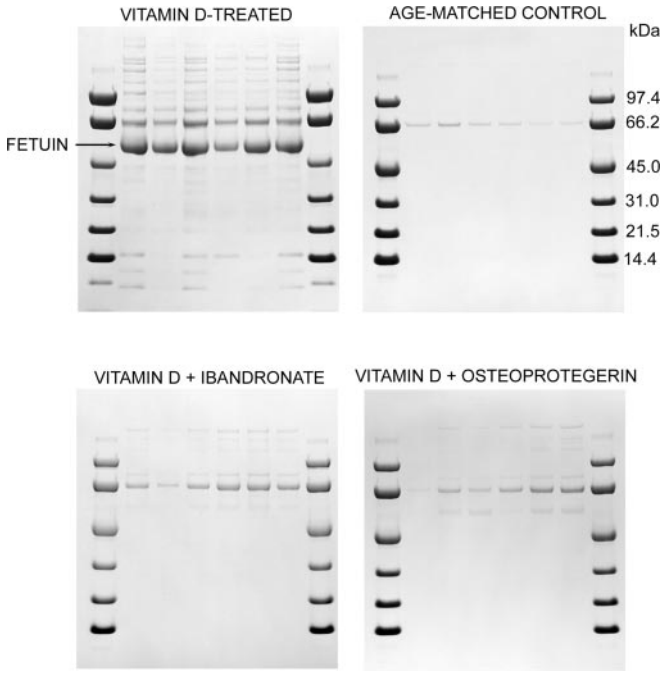

FIG. 2. **SDS-gel electrophoresis of the pellet fraction obtained by centrifugation of serum from rats treated with vitamin D alone, with vitamin D plus ibandronate, and with vitamin D plus osteoprotegerin.** Serum samples were obtained at death from the animals in the experiment described in the legend to Fig. 1 and 200-μl aliquots of each were centrifuged for 2 h at 16,000 $\times$ *g* to sediment any fetuin-mineral complex that might be present. The resulting pellet fractions were dissolved in 40 μl of SDS loading buffer containing 60 mM EDTA, pH 7.5, and 20 μl of each sample was electrophoresed on a 4–12% gradient gel and stained with Brilliant Blue R.

lanes from rats treated with vitamin D alone were not present in sufficient abundance to permit identification by N-terminal protein sequencing, and there was too little MGP in the fetuin-mineral complex (Fig. 1) for its presence to be detected in these electrophoresis experiments.

As also seen in Fig. 2, the 59-kDa fetuin band is completely absent in the lanes for the 6 control samples, and dramatically reduced in the lanes for the 6 rats treated with vitamin D plus ibandronate and for the 6 rats treated with vitamin D plus osteoprotegerin. These results are in excellent agreement with the levels of fetuin measured in the pellet fractions of the different treatment groups by radioimmunoassay (Fig. 1).

Serum was also analyzed to determine the total serum levels of cross-linked N-telopeptides, a specific biochemical marker for bone resorption activity, and total serum levels of calcium, phosphate, fetuin, and albumin. As seen in Fig. 3, treatment with vitamin D alone elevated bone resorption activity by over 2-fold ($p < 0.0001$ when compared with control rats), reduced serum fetuin levels by 65% ($p < 0.0001$), and reduced serum albumin levels by 15% ($p < 0.001$). As also seen in Fig. 3, treatment with vitamin D plus ibandronate or with vitamin D plus osteoprotegerin reduced bone resorption activity to below control values, and prevented the vitamin D-induced reduction in serum fetuin and serum albumin. In agreement with earlier studies (4, 6), serum calcium levels were elevated by a comparable 35% ($p < 0.001$ when compared with control rats) in the rats treated with vitamin D alone, with vitamin D plus ibandronate, and with vitamin D plus osteoprotegerin (data not shown).

Thoracic aortas from 6 rats in each treatment group were extracted with acid to dissolve any mineral that might be present, and the resulting extracts were analyzed for calcium, phosphate, fetuin, and MGP. In agreement with earlier studies (1, 3, 4, 6), these analyses showed that phosphate and MGP levels were elevated by over 10-fold in the aortas of rats treated with vitamin D alone, and that calcium levels were elevated by over 5-fold. Calcium, phosphate, and MGP levels were not significantly above control levels ($p > 0.05$) in rats treated with vitamin D and either ibandronate or osteoprotegerin. Fetuin levels in the thoracic aorta of rats treated with vitamin D alone were not significantly different from fetuin levels in the aorta of control rats or of rats treated with vitamin D and either ibandronate or osteoprotegerin ($p > 0.4$ for each comparison) (data not shown).

Correlation between Artery Calcification and Serum Levels of the Fetuin-Mineral Complex in Rats Treated for Different Times with Vitamin D—Additional experiments were carried out to determine whether the timing of vitamin D-induced artery calcification correlates with the appearance of the fetuin-mineral complex in serum and with the reduction in total serum fetuin levels. The timing of artery calcification was first evaluated by Alizarin red staining and by extraction and analysis of calcium phosphate. This experiment showed that there was no detectable Alizarin red staining for calcification in the abdominal aorta of the 6 rats examined at 24 or 48 h, and extensive Alizarin red staining for calcification in the abdominal aorta of 1 of the 6 rats examined at 72 h, in 5 of the 6 rats examined at 84 h, and in all of the rats examined at 96 h. Fig. B (see Supplemental Materials) shows representative Alizarin red staining in the abdominal aortas of rats treated with vitamin D for 48, 72, 84, and 96 h. Chemical analyses further showed that there was significant accumulation of calcium and phosphate in each of the abdominal aortas that stained for calcification with Alizarin red, and no detectable accumulation of calcium and phosphate in the abdominal aortas that did not stain (see Fig. 4). For the aortas with significant evidence of calcium phosphate accumulation, the increase in calcium was significantly correlated with the increase in phosphate ($R^2 = 0.993$).

The possible presence of the fetuin-mineral complex in the serum obtained from each rat was evaluated by biochemical analysis of the pellets formed by centrifuging serum for 2 h at 16,000 $\times$ *g*. A translucent, compact pellet was found in each of the 6 serum samples from animals treated with vitamin D for 84 or 96 h, but could not be detected in the serum from the 6 rats treated with vitamin D for 24, 48, or 72 h. After removal of the supernatants, 0.15 M HCl was added to each tube to dissolve any fetuin-mineral complex that might be present, and the resulting acid extracts (termed "pellet fractions") were analyzed to determine the levels of calcium, phosphate, and fetuin. As seen in Fig. 5, there was no significant increase in calcium, phosphate, or fetuin in the pellet fractions from the rats treated with vitamin D for 24 h when compared with the corresponding pellet fractions in untreated control rats. There was, however, a statistically significant increase in calcium, phosphate, and fetuin in the pellet fractions from rats treated with vitamin D for 48 and 72 h ($p \leq 0.05$ when compared with control rats), and a further dramatic increase in calcium, phosphate, and fetuin in the pellet fractions from rats treated with vitamin D for 84 and 96 h ($p \leq 0.001$ when compared with control rats). As can be seen in Fig. 5, there is a relatively large standard deviation for the data obtained at the 48- and 72-h time points. Further analysis of the data at these time points showed that the amount of calcium recovered in the pellet fractions (which ranged from 5 to 65 nmol) correlated with the amount of phosphate ($R^2 = 0.996$) and with the amount of fetuin ($R^2 = 0.862$). These correlations show that all three parameters vary in parallel from one serum sample to another, and therefore indicate that the large standard deviation seen in the data at the 48- and 72-h time points is because of variable

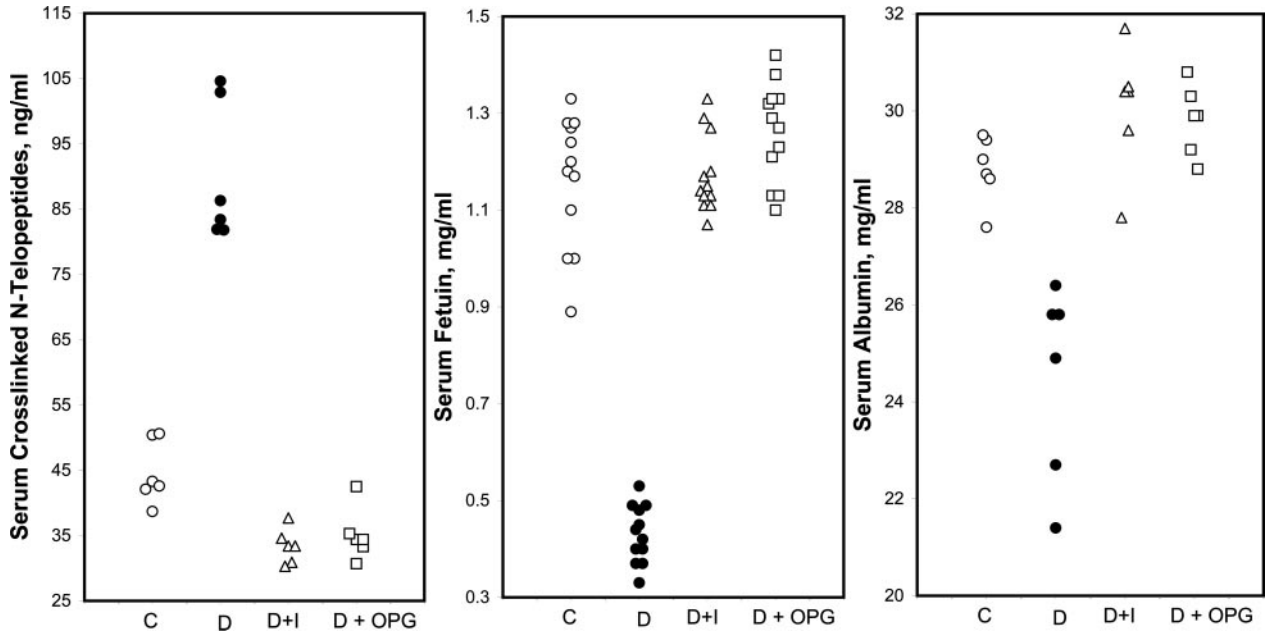

FIG. 3. **Elevated levels of bone resorption activity and depressed levels of serum fetuin and serum albumin are seen in rats treated with vitamin D alone, but not in rats treated with vitamin D plus ibandronate or with vitamin D plus osteoprotegerin.** Serum samples were obtained at death from the 24 animals in the experiment described in the legend to Fig. 1 and analyzed to determine the level of cross-linked N-telopeptides (OSTEOMARK NTx), a specific marker for bone resorption activity,[2] and to determine the level of serum albumin. Serum samples from the 24 animals in the experiment described in the legend to Fig. 1 and from 24 animals in an identical repeat experiment were analyzed by radioimmunoassay to determine the level of rat fetuin (see "Experimental Procedures"). *C*, age-matched control rats; *D*, rats treated with vitamin D alone; *D + I*, rats treated with vitamin D plus ibandronate; *D + OPG*, rats treated with vitamin D plus osteoprotegerin.

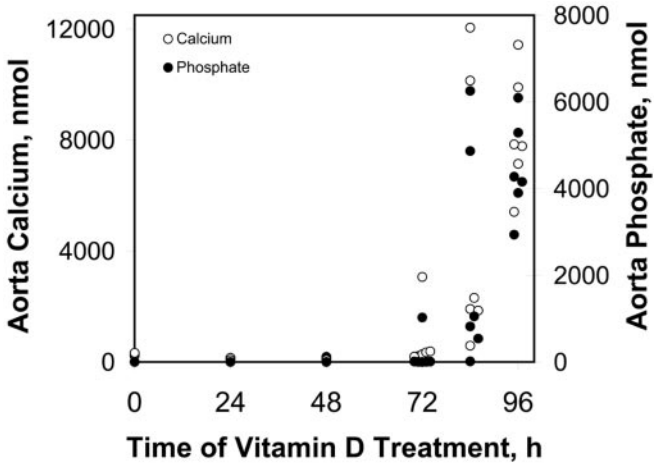

FIG. 4. **Time course of calcification in the abdominal aortas of rats treated with vitamin D.** Six 7-week-old male rats served as untreated controls ($t = 0$); 6 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$ and were killed at $t = 24$ h; 6 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$ and 24 h and were killed at $t = 48$ h; and 18 received 500,000 IU of vitamin D_3/kg body weight at $t = 0$, 24, and 48 h, 6 of these were killed at $t = 72$, 6 at $t = 84$, and 6 at $t = 96$ h. The abdominal aorta and attached arteries were dissected as a unit from each animal, stained for calcification with Alizarin red S, and then extracted with 150 mM HCl to dissolve any calcification that might be present. The resulting acid extracts were analyzed for calcium and phosphate (see "Experimental Procedures").

amounts of the fetuin-mineral complex in these 12 serum samples and not to methodological problems with the analysis of the components of the complex.

Total serum fetuin levels were also measured in each serum sample to determine whether the timing of the decrease in serum fetuin correlates with artery calcification and the appearance of the fetuin-mineral complex in serum. As seen in Fig. 6, total serum fetuin levels were not significantly reduced at 24 or 48 h of vitamin D treatment, but were reduced by 22% at 72 h ($p \leq 0.01$ compared with $t = 0$) and by 80% at 84 and 96 h ($p \leq 0.0001$ compared with $t = 0$).

DISCUSSION

The objective of the present study was to characterize the biochemical mechanism responsible for the linkage between bone resorption and artery calcification, a linkage whose existence is strongly supported by the fact that specific inhibitors of bone resorption potently inhibit artery calcification (1, 4, 5). Our working hypothesis is that there is a causative agent for artery calcification that arises in bone resorption, travels in blood, and then induces calcification in the artery wall. In the present study we have directly tested this blood born theory by analyzing blood levels of one agent in blood that is plausibly associated with artery calcification, the serum fetuin-mineral complex, in animals in which artery calcification has been induced by treatment with vitamin D doses that increase bone resorption, and inhibited by doses of ibandronate and osteoprotegerin that inhibit this vitamin D-induced increase in bone resorption.

These tests show that the fetuin-mineral complex is indeed in the blood of vitamin D-treated rats with on-going artery calcification, and not in the blood of rats in which bone resorption (and artery calcification) have been inhibited by ibandronate or osteoprotegerin. These tests further show that there is a dramatic, 3-fold reduction in circulating fetuin levels in rats with on-going artery calcification, and no reduction in fetuin levels in rats in which artery calcification has been inhibited by ibandronate or osteoprotegerin. We speculate that these two observations are linked, and that the reduced total serum fetuin levels seen in animals treated with vitamin D alone is caused by the clearance of the fetuin-mineral complex from serum. This hypothesis is supported by the fact that clearance of the fetuin-mineral complex from the blood of the etidronate-treated rat is also associated with the reduction in total blood levels of fetuin (9).

These experiments also show that there is a significant, 15%

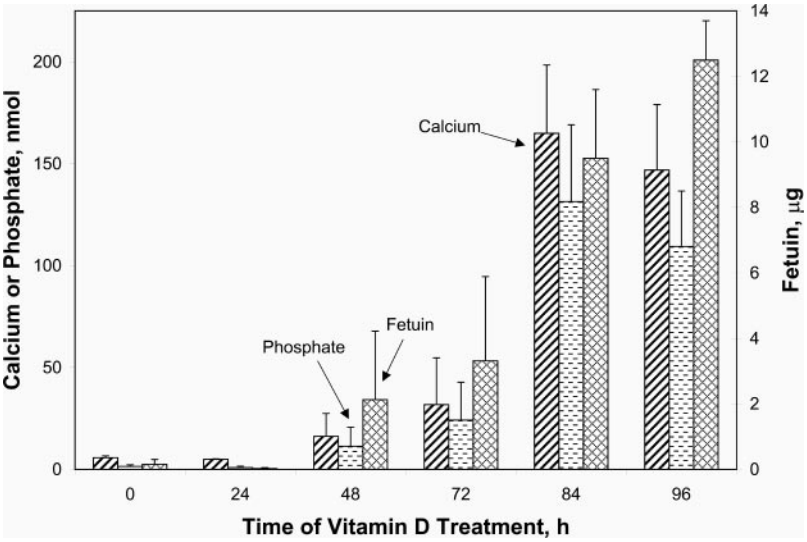

FIG. 5. **Time course of the appearance of the fetuin-mineral complex in the serum of rats treated with vitamin D.** Serum was obtained at death from each rat in the experiment described in the legend to Fig. 4, and a 200-μl aliquot of serum from each rat was centrifuged for 2 h at 16,000 $\times$ *g* to sediment any fetuin-mineral complex that might be present. The supernatant was removed, 0.15 M HCl was added to each tube to dissolve the fetuin-mineral complex, and the acid extracts were analyzed for calcium, phosphate, and fetuin (see "Experimental Procedures"). The figure shows the mean $\pm$ S.D. for the values obtained for the serum of the 6 rats killed at each time point and for 6 untreated control animals (shown as $t = 0$).

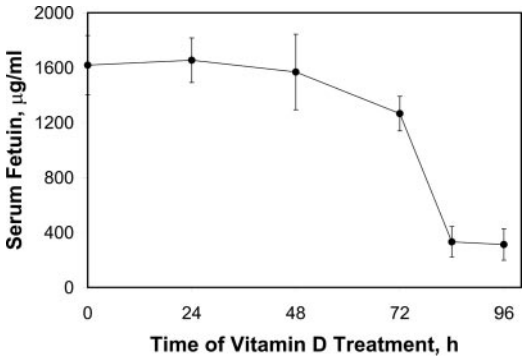

FIG. 6. **Time course of the decrease in fetuin in the serum of rats treated with vitamin D.** Serum was obtained at death from each rat in the experiment described in the legend to Fig. 4 and analyzed by radioimmunoassay to determine the level of rat fetuin (see "Experimental Procedures"). The figure shows the mean $\pm$ S.D. for the serum fetuin levels in the 6 rats killed at each time point and for 6 untreated control animals (shown as $t = 0$).

reduction in serum albumin in rats treated with vitamin D. Because albumin is the second most abundant protein component of the fetuin-mineral complex pellet obtained by centrifuging serum from vitamin D-treated rats (Fig. 2), it is tempting to speculate that this 15% reduction in serum albumin is caused by clearance of the fetuin-mineral complex from serum. This hypothesis is supported by the fact that treatment with vitamin D plus either ibandronate or osteoprotegerin eliminates detectable fetuin-mineral complex from serum as well as the reduction in total serum albumin (Fig. 3). If the clearance of the fetuin-mineral complex from serum indeed causes the reduction in total serum albumin seen in vitamin D-treated rats, albumin must be physically associated with the fetuin-mineral complex, and the consistent presence of albumin in the fetuin-mineral complex pellet (7, 9, 24) reflects specific binding of albumin to the complex rather than, as previously suggested (7, 9, 24), the nonspecific contamination of the fetuin-mineral complex pellet with serum.

Subsequent experiments were carried out to determine whether the timing of vitamin D-induced artery calcification correlates with the appearance of the fetuin-mineral complex in serum and with the reduction in total serum fetuin levels. These experiments revealed that the onset of artery calcification occurs within an unusually narrow window of vitamin D treatment, with no calcification in the 6 rats examined at 24 and 48 h, calcification in 1 of 6 rats examined at 72 h, calcification in 5 of 6 rats examined at 84 h, and calcification in all rats examined at 96 h. These results support a model in which calcification occurs rapidly and extensively throughout the artery wall whenever a critical threshold level of the putative blood borne agent is reached, and show that this threshold is reached between 48 and 72 h in 1 rat, between 72 and 84 h in 4 rats, and between 84 and 96 h in 1 rat.

In this time course experiment, significant levels of the serum fetuin-mineral complex were first found at 48 h, were further increased at 72 h, and were maximally elevated at 84 and 96 h. These observations show that the onset of artery calcification (between 72 and 84 h for 4 of 6 rats) is linked to the timing of maximal elevations in serum fetuin-mineral complex levels (between 72 and 84 h for 5 of 6 rats). It is of interest to note that the one rat with artery calcification at the 72-h time point also was the rat with the highest levels of the serum fetuin-mineral complex at 72 h, a level that was within the range of values found in rats at the 84- and 96-h time points.

A significant decline in serum fetuin was first observed at 72 h and reached maximal levels at 84 and 96 h. These observations demonstrate that the decline in total serum fetuin observed at a given time point is closely correlated with the absolute level of the fetuin-mineral complex in serum. Whereas it seems likely that the massive decline in serum fetuin seen in the 72–84-h interval is caused by rapid formation and clearance of the fetuin-mineral complex from serum, it should be noted that the 60 μg/ml of total serum fetuin associated with the complex at 84 h would have to be formed and cleared every 45 min to account for the 1 mg/ml of total fetuin that is lost from serum in the 72–84-h time period. Further studies will clearly be needed to establish the rate of formation and clearance of the fetuin-mineral complex from the serum of the vitamin D-treated rat.

Whereas the correlation of the fetuin-mineral complex with artery calcification induced by vitamin D treatment supports the blood born theory of artery calcification, the biochemical linkage between the presence of the fetuin-mineral complex in blood and artery calcification is not yet clear. The existing biochemical and genetic evidence strongly support the hypothesis that the function of fetuin is to inhibit mineralization, not promote it, and that the fetuin-mineral complex is formed as part of the mechanism by which crystal nuclei are trapped and subsequently cleared from blood. How then is it possible that high levels of the fetuin-mineral complex are so predictive of artery and soft tissue calcification in rats treated with toxic doses of vitamin D? One possibility is that the excessive bone resorption induced by vitamin D generates such massive

amounts of the fetuin-mineral complex that the ability of fetuin to arrest the growth of the mineral component is impaired. If the fetuin-mineral complex were to occasionally enter the artery wall, the impaired ability of fetuin to arrest the growth of the mineral component could then induce artery calcification by a process of seeded crystal growth. Another possibility is that the fetuin-mineral complex is formed as a downstream product of a pathway that begins with the agent that actually causes artery calcification, and that levels of the fetuin-mineral complex in serum are markers for the level of the true causative agent for vitamin D-induced artery calcification.

The blood-born theory for the initiation of artery calcification in the vitamin D-treated rat model predicts that the increased tendency to calcify could be systemic rather than restricted to arteries. There is evidence that this is the case. The doses of vitamin D used in the present study to induce artery calcification also cause extensive calcification of kidneys and lungs, and the calcification of these tissues is completely prevented by doses of ibandronate and osteoprotegerin that inhibit bone resorption (4, 6). Vitamin D administration has also been known for many years to sensitize animals for calcification at sites of injury or injection with challenger, the so-called calciphylactic response (31). We have recently shown that the calciphylactic response to epilation and to $FeCl_3$ injection in vitamin D-treated rats is also completely prevented by doses of ibandronate that inhibit bone resorption (32). The systemic nature of the soft tissue calcifications found in rats treated with vitamin D, and the ability of bone resorption inhibitors to consistently prevent these calcifications, are strong support for the blood-born theory for the initiation of ectopic calcifications in the vitamin D-treated animal.

Whereas the present studies demonstrate an association between the fetuin-mineral complex in blood and artery calcification induced by vitamin D treatment, it remains to be demonstrated that there is a similar association between the presence of a fetuin-mineral complex in blood and the occurrence of artery calcification in other circumstances. It is of interest to note, however, that uremic patients with calciphylaxis have a reduction in total serum fetuin (33), and that low serum fetuin concentrations are correlated with cardiovascular mortality in dialysis patients (34). These reductions in serum fetuin could be associated with accelerated formation and clearance of the fetuin-mineral complex from blood. Studies are in progress to improve the sensitivity of the fetuin-mineral complex assay to the point at which its presence can be reliably detected in warfarin-treated and control rats and in humans with various degrees of artery and soft tissue calcification.

Previous studies have shown that the dose of vitamin D used in the present study is lethal (6), and it is intriguing to note that the dramatic, 3–4-fold reduction in serum fetuin was found in serum obtained at 96 h after this vitamin D dose (Figs. 3 and 6), and that death occurs between 96 and 120 h (6). The striking correlation between depletion of serum fetuin and death suggests that exhaustion of serum fetuin may be a significant factor in the pathophysiology of vitamin D toxicity. This possibility is supported by the observation that ibandronate doses that prevent artery calcification also prevent both the reduction in serum fetuin (Fig. 4) and death (6). Studies are currently in progress to isolate sufficient amounts of the native, fully phosphorylated form of rat fetuin (9) to test the ability of fetuin injections to inhibit ectopic calcifications and prevent death in rats treated with toxic doses of vitamin D.

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