The Elastic Lamellae of Devitalized Arteries Calcify When Incubated in Serum

Evidence for a Serum Calcification Factor

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Objective—To determine whether serum contains an activity that induces artery calcification.

Methods and Results—The elastic lamellae of devitalized rat aortas calcify rapidly in rat or bovine serum, or in human serum provided [Pi] ≥2 mmol/L. This calcification is attributable to a potent serum calcification factor (SCF), one that causes devitalized aortas to calcify when incubated in DMEM containing as little as 1.5% serum but not in DMEM alone. The SCF that initiates medial elastin calcification has the same 50- to 150-kDa size and protease sensitivity as the SCF shown previously to initiate calcification of type I collagen. Our working hypothesis is that the same SCF initiates calcification of collagen and elastin, and that this SCF arises from sites of normal bone mineralization and, like alkaline phosphatase, is released into general circulation. The SCF does not initiate medial elastin calcification in living arteries, which suggests that vascular cells may prevent this calcification. This hypothesis is supported by the observations that living arteries secrete the calcification inhibitor matrix Gla protein (MGP); that inactivation of MGP with warfarin causes living arteries to calcify; and that addition of MGP to medium containing warfarin prevents this calcification.

Conclusion—The elastic lamellae of devitalized aortas calcify rapidly in serum. (***Arterioscler Thromb Vasc Biol*. 2006; 26:1079-1085.**)

Key Words: medial artery calcification ■ elastic lamellae ■ serum calcification factor ■ matrix Gla protein ■ devitalized and living arteries

Two major types of arterial calcification have been observed in human patients.[1,2] One affects the intimal layer of arteries and occurs within atherosclerotic plaques. The other involves the artery media and initially occurs within the elastic lamellae. This second type of vascular calcification is common in patients with chronic kidney disease and in patients with diabetes mellitus. Each type of arterial calcification has different physiological consequences, with clear-cut evidence for adverse hemodynamic changes attributable to medial wall calcification but not to atherosclerotic plaque calcification, and the possible contribution of atherosclerotic plaque calcification to plaque rupture and subsequent thrombosis, an issue that does not apply to medial wall calcification.

Our long-term goal is to understand the mechanisms that initiate calcification of the elastic lamellae of the artery media and the mechanisms that inhibit this calcification. In the course of our investigations, we became intrigued with the evidence for an association between bone metabolism and artery calcification,[3] an association that led us to propose that medial artery calcification is linked to bone resorption. One prediction of this hypothesis is that inhibitors of bone resorption should inhibit artery calcification.[3] In previous studies, we tested this prediction using 3 different types of bone resorption inhibitors, each with an entirely different mode of action on the osteoclast, the amino bisphosphonates alendronate and ibandronate,[3–5] the cytokine osteoprotegerin,[6] and the V-H^+-ATPase inhibitor SB 242784.[7] Each bone resorption inhibitor proved to potently inhibit medial artery calcification in both of the rat models tested, rats in which the calcification inhibitory activity of matrix Gla protein (MGP) was removed by treatment with warfarin and rats treated with high doses of vitamin D.

Ibandronate, osteoprotegerin, and SB 242784 are all highly specific inhibitors of the osteoclast at the concentrations used in these studies and have no known actions on vascular cells. Their ability to potently inhibit artery calcification therefore strongly supports the hypothesis that medial artery calcification is linked to bone resorption. Although the nature of the link between bone metabolism and artery calcification has not yet been established, we recently proposed the bloodborne theory for artery calcification to account for this link, the theory that a causative agent (or agents) for artery calcification arises in bone metabolism, travels in blood, and then

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induces calcification in the elastic lamellae of the artery media.[3] This theory predicts that serum contains a causative agent for medial artery calcification, and that the elastic lamellae of the artery media should therefore calcify when devitalized arteries are incubated in serum.

It has been known for >40 years that devitalized rat aortas do indeed calcify when incubated in rat serum at 37°C.[8,9] This calcification is confined to the elastic lamellae of the aorta media, and examination of the calcified tissue by electron diffraction and electron microscopy has shown that the calcification consists of apatite-like crystals localized almost exclusively within elastin fibers.

The objectives of the present experiments were to confirm that incubation of devitalized rat aortas in rat serum does indeed cause calcification within the elastic lamellae of the aorta media, and to determine whether a similar activity is also found in bovine and human serum. Arteries did calcify during incubation in rat, bovine, and human serum, and additional experiments were accordingly performed to compare the biochemical characteristics of the serum calcification factor responsible for medial artery calcification with the recently described serum calcification factor that initiates the calcification of the type I collagen,[10] and to assess the ability of living arteries in organ culture to resist serum-initiated medial calcification.

Materials and Methods

The methods used in these studies are available in the online supplement at http://atvb.ahajournals.org, and only a brief synopsis of these methods is presented here. The devitalized artery calcification assay used here was initially developed to investigate the recalcification of demineralized bone.[10,11] In brief, EDTA-devitalized rat aortas are placed in rat or human serum, or in DMEM ([Pi]=2 mmol/L) containing different amounts of serum, and incubated at 37°C and 5% CO_2. Tissue calcification was evaluated by staining of whole arteries with Alizarin red, staining of histological sections by von Kossa, and quantification of calcium and phosphate in acid extracts of the arteries using procedures described previously.[10,11] Procedures for characterizing the serum factor that induces artery calcification have also been published previously.[10,11]

To determine the effect of living cells on medial calcification, thoracic aortas were removed from rats within 15 minutes of death and separately incubated for 6 days in 10 mL of DMEM containing 15% FBS. To determine the effect of warfarin and MGP on medial calcification in arteries containing living cells, carotid arteries were removed from rats within 15 minutes of death, cut into 3- to 4-mm segments, and placed separately in culture dishes containing 2 mL of DMEM or 2 mL DMEM plus 15% FBS. Some dishes were treated with sodium warfarin at a dose of 10 μg/mL, whereas other dishes were treated with this warfarin dose together with 30 or 100 μg/mL purified bovine MGP. MGP was added as an aliquot of 5 mg/mL MGP in 50 mmol/L HCl, with continuous swirling to rapidly disperse the protein in the culture medium.

Results

Incubation of EDTA-Devitalized Rat Aortas in Rat Serum Causes Medial Artery Calcification In Vitro

The initial test to determine whether serum contains a causative agent for artery calcification was performed using rat thoracic aortas that had been first freed of any possible endogenous mineral by extraction for 72 hours with 0.5 mol/L EDTA, pH 7.5, a procedure that also killed all cells originally found in the artery. Each devitalized aorta was placed in 2-mL rat serum and incubated for 6 days at 37°C. As seen in Figure 1, ≈3.5 μmol of serum calcium was taken up by the aorta over the 6-day incubation, with half of the uptake occurring in the first day of incubation. After incubation in rat serum for 6 days, Alizarin red staining of the thoracic aortas revealed the presence of extensive calcification throughout most of the thoracic aorta and the smaller branch arteries, and von Kossa staining of aorta sections showed that this calcification is associated with the elastic lamellae of the artery media (Figure 2). Chemical analysis showed that incubation of devitalized aortas for 6 days in rat serum also increased aorta levels of calcium and phosphate (supplemental Table I, available online at http://atvb.ahajournals.org.); the amount of calcium recovered from the aorta accounted for the 3.5 μmol of serum calcium taken up by the aorta during the 6-day incubation (Figure 1). Comparable artery calcification was also seen after incubation for 6 days

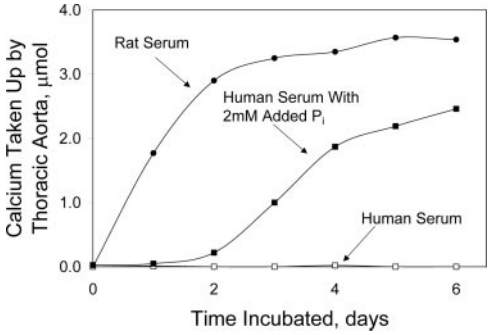

Figure 1. Time course of calcium uptake by devitalized aortas during incubation in rat or human serum. Rat thoracic aortas were treated with 0.5 mol/L EDTA, pH 7.5, and then incubated for 6 days at 37°C in 2 mL rat serum (ion product: 1.2 mmol/L ionic Ca×3.2 mmol/L Pi=3.8 $mmol^2/L^2$), 2 mL adult human serum (ion product: 1.2 mmol/L ionic Ca×1.2 mmol/L Pi=1.4 $mmol^2/L^2$), or 2 mL of adult human serum, in which the phosphate concentration had been increased to 3.2 mmol/L by the addition of 0.5 mol/L sodium phosphate buffer, pH 7.4 (ion product: 3.8 $mmol^2/L^2$). Calcium uptake was determined from the decrease in serum calcium attributable to the presence of the devitalized aorta. This experiment has been repeated twice with comparable results.

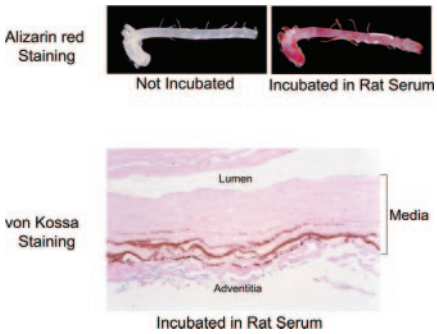

Figure 2. Calcification of devitalized rat aortas by incubation for 6 days in rat serum. After incubation for 6 days in rat serum (Figure 1 legend), the devitalized thoracic aortas were either stained for calcification with Alizarin red (stains calcification red) or fixed in formalin, cut in 5-μm sections, and stained for calcification with von Kossa (stains calcification brown to black) and counterstained with nuclear fast red (stains elastin and cytoplasm pink and nuclei red).

in heparinized rat plasma, FBS, or newborn calf serum (data not shown).

Evidence That the Elastic Lamellae of the Artery Media, Not Cell Debris, Is the Location of Calcification in Arteries That Have Been Incubated in Serum

To evaluate the possible effect of vascular cell debris on serum-initiated medial artery calcification, the EDTA-treated aortas were subsequently extracted with acetone for 2 hours to remove lipid and with 6 mol/L guanidine HCl for 24 hours to solubilize protein. Histological examination of aorta cross-sections showed that these additional extraction steps did indeed remove all detectable cellular debris. However, in spite of the complete absence of cellular debris in the artery media, the aortas still stained extensively with Alizarin red after incubation for 6 days in serum, and calcium and phosphate were still taken up by the aortas (supplemental Table II); the location of calcification was again restricted to the elastic lamellae of the aorta media (data not shown). In another test, elastin fibers were purified from the media of bovine aortas using procedures that remove all vascular cell debris as well as collagen;[12,13] as can be seen in supplemental Table II, this purified aortic elastin is also extensively calcified by incubation for 6 days in rat serum.

As a final test, devitalization procedures were used that intentionally preserved some or most of the cellular debris and did not involve EDTA extraction. As seen in supplemental Table II, devitalization by fixation in formalin or by lyophilization had no significant impact on the quantitative extent of calcium and phosphate uptake by the aortas during incubation in serum for 6 days. Despite the presence of the formalin-fixed vascular cells in the aorta wall, von Kossa staining for calcification was again restricted to the elastic lamellae of the aorta media, with no evidence of calcification associated with vascular cells or other structures between the lamellae (Figure 3; a hematoxylin/eosin–stained section from the same sample is shown in supplemental Figure I). This lamellar calcification appears as discrete brown calcification foci that range from 0.5 to 5 μm in size, each probably representing separate nucleation events.

Biochemical Characterization of the Serum Activity Responsible for Calcifying Elastic Lamellae in the Artery Media

Additional experiments were performed to further characterize the biochemical activity that is responsible for the calcification of the elastic lamellae of devitalized arteries during incubation in serum. The initial experiments were conducted to determine whether the elastic lamellae of devitalized aortas calcify when incubated in DMEM containing a calcium phosphate ion product (1.8 mmol/L [Ca]$\times$2 mmol/L [Pi]=3.6 mmol^2/L^2) comparable to the ion product of rat serum (1.2 mmol/L [Ca]$\times$3.2 mmol/L [Pi]=3.8 mmol^2/L^2). Devitalized arteries did not calcify when incubated in this modified DMEM solution alone but did consistently calcify if the DMEM contained $\geq$1.5% serum (Table). Further experiments showed that DMEM must contain a physiological calcium phosphate ion product

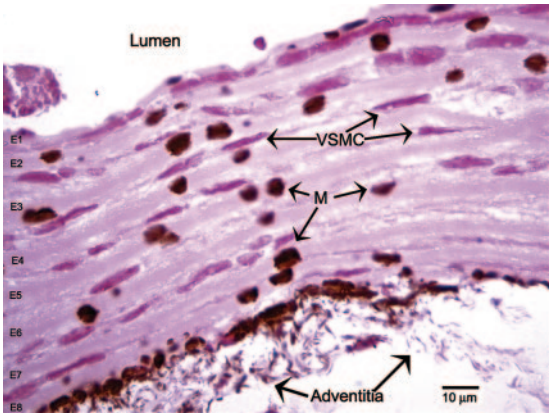

Figure 3. Effect of formalin fixation on the histological location of serum-induced artery calcification. Rat thoracic aortas were placed in formalin within 15 minutes of death and fixed for 3 days. A 2-cm segment of each aorta was then washed with water and incubated in 2 mL of rat serum for 6 days at 37°C. Five-micron sections were stained by von Kossa and counter-stained by nuclear fast red. $\times$1000 magnification; Bar=10 μm. VSMC indicates vascular smooth muscle cells; M, calcification foci. Note that calcification is restricted to the 8 elastic lamellae (E1 to E8 in image).

(3.6 mmol^2/L^2, achieved by increasing phosphate in DMEM to 2 mmol/L) for artery calcification to occur in the presence of added serum; no calcification occurs in basal DMEM (1.8 mmol/L [Ca]$\times$0.9 mmol/L [Pi]=1.6 mmol^2/L^2) even in the presence of 15% serum.

This phosphate-boosted DMEM ([Pi]=2 mmol/L) was used in biochemical characterization experiments, the results of which are summarized in the Table. These experiments show that the serum activity responsible for calcification of medial elastin fibers in devitalized arteries has an apparent gel filtration molecular weight of 50 to 150 kDa (supplemental Figures II and III), is inactivated by digestion with trypsin or chymotrypsin (supplemental Figure IV), and introduces a mineral phase into the elastin fiber that has an apatite-like X-ray diffraction pattern (supplemental Figure V). The biochemical properties of the serum activity responsible for

Comparison of the Serum Activities That Initiate the Calcification of Demineralized Bone and Devitalized Arteries

	Demineralized Bone	Devitalized Artery
Molecular weight (Gel filtration)	50–150 kDa	50–150 kDa
Protease sensitivity	Trypsin inactivates Chymotrypsin inactivates	Trypsin inactivates Chymotrypsin inactivates
Dose dilution of activity:		
DMEM with $\geq$1.5% serum	Calcification	Calcification
DMEM with $\leq$0.15% serum	No calcification	No calcification
Mineral type (X-ray diffraction)	Apatite-like	Apatite-like
Location of mineral	Collagen fibers	Elastin fibers

Demineralized bone data are from Price et al,[10] and devitalized artery data are from experiments presented in the online data supplement.

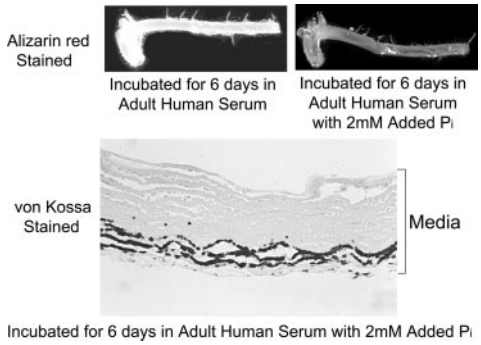

Figure 4. Calcification of devitalized aorta by incubation for 6 days in human serum. After incubation for 6 days in human serum or in human serum containing 2 mmol/L added Pi (Figure 1 legend), the devitalized thoracic aortas were either stained for calcification with Alizarin red or fixed in formalin, cut in 5-μm thick sections, and stained for calcification with von Kossa and counterstained with nuclear fast red.

calcification of medial elastin in serum is compared in the Table to the previously identified serum activity that initiates calcification in type I collagen of tendon and demineralized bone in serum.[10] A consistent feature of the calcification of tendon, demineralized bone, or devitalized arteries during incubation in neat serum is the apparent absence of calcification associated with cellular debris, even when this debris is intentionally preserved by formalin fixation. Calcification is instead always within the fibers of collagen[10] or elastin (Figure 3).

Calcification of Devitalized Rat Aortas by Incubation in Human Serum

Experiments were performed to determine whether devitalized aortas will also calcify after incubation in human serum. As seen in Figure 4, no aorta calcification could be detected by Alizarin red staining after incubation for 6 days in adult human serum, and analysis of calcium levels in serum during incubation failed to provide evidence for calcium uptake by the aorta (Figure 1). Because the level of phosphate in this human serum sample was 1.2 mmol/L, whereas the level of phosphate in the rat serum used in the above experiments was 3.2 mmol/L, a possible explanation for the failure of aortas to calcify in adult human serum could be that the level of phosphate is too low. To test this possibility, phosphate was added to adult human serum to achieve a net increase in phosphate concentration of 2 mmol/L. Incubation of aortas in this phosphate-boosted human serum did cause uptake of serum calcium by the aorta over the 6-day incubation period (Figure 1) as well as extensive Alizarin red staining for calcification (Figure 4) and accumulation of calcium and phosphate in the artery (supplemental Table I). Similar results were seen on histological examination of von Kossa–stained aorta sections, with extensive focal staining of the elastic lamellae in the media of aortas incubated for 6 days in adult human serum in which the phosphate concentration had been increased by 2 mmol/L (Figure 4) but not in aortas incubated in adult human serum alone (data not shown).

To better understand the role of serum phosphate concentration in artery calcification, devitalized thoracic aortas were incubated for 6 days in adult human serum containing

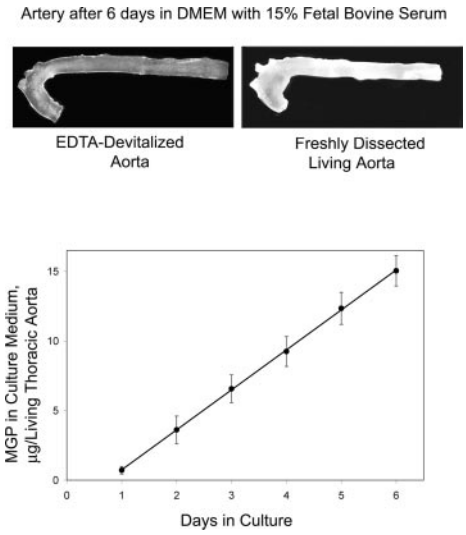

Figure 5. Living rat arteries secrete the calcification inhibitor MGP and are resistant to serum-induced medial artery calcification. Thoracic aortas were removed from rats within 15 minutes of death and placed in organ culture with 10 mL of DMEM (2 mmol/L Pi) containing 15% FBS. EDTA-devitalized rat thoracic aortas were placed in the same medium as a positive calcification control. Aortas were then incubated for 6 days at 5% CO_2 and 37°C. Top, Alizarin red staining for calcification in representative living and EDTA-devitalized thoracic aortas. Bottom, Cumulative MGP production by living thoracic aortas as determined by radioimmunoassay of the cell culture medium.[24] Each point shows the average MGP production in the 6 aortas examined, and the error bars show the SDs.

different amounts of added phosphate and calcification was assessed by quantitative analysis of the amount of calcium and phosphate incorporated into the aorta. As seen in supplemental Figure VII, there is a sigmoid dependence of artery calcification on the concentration of phosphate in adult human serum, with a threshold for artery calcification between 1.5 and 2 mmol/L phosphate.

Serum-Induced Medial Artery Calcification Does Not Occur if Viable Cells Remain in the Artery Wall

To evaluate the possible role of vascular cells in regulating serum-induced medial calcification, 3-cm segments of rat thoracic aorta were removed immediately after death and separately placed into 10 mL of DMEM supplemented with 15% FBS and incubated at 37°C for 6 days. There was no detectable Alizarin red staining in any of the 6 living thoracic aortas examined after incubation for 6 days in this organ culture medium, whereas there was extensive staining in each of the 6 devitalized aortas; examples of the Alizarin red staining seen in this experiment are shown in Figure 5. Examination of hematoxylin/eosin–stained sections of the living aortas after the 6-day incubation showed that vascular cell morphology was indistinguishable from that seen in thoracic aortas examined immediately after death, a result that is in agreement with observations made in previous studies of rat arteries in organ culture.[14,15] These results demonstrate that the elastic lamellae of the artery media fail to calcify if living cells remain in the vascular wall.

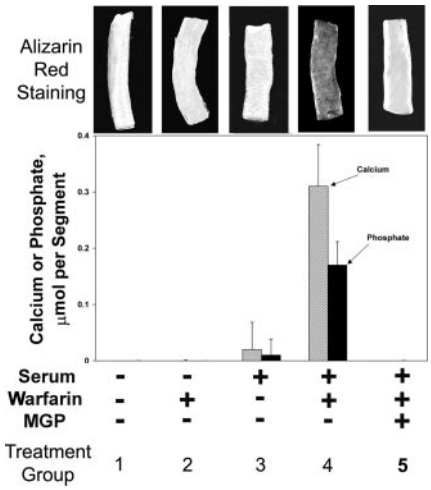

Figure 6. Living rat arteries calcify when incubated in culture medium containing serum and warfarin, and this calcification is prevented by the addition of purified MGP. Carotid arteries were removed from rats within 15 minutes of death and cut into 3- to 4-mm segments; each segment was immediately placed in culture dishes containing 2 mL of DMEM (2 mmol/L Pi) or 2 mL DMEM plus 15% FBS. Some dishes were treated with 10 μg/mL sodium warfarin, whereas other dishes were treated with 10 μg/mL warfarin together with 100 μg/mL purified bovine MGP. Arteries were incubated for 4 days at 5% CO_2 and 37°C, and then stained for calcification with Alizarin red and analyzed for calcium and phosphate. There were 24 carotid artery segments in all groups except the MGP-treated group, which had 12. The figure shows representative examples of Alizarin red staining and the mean and SD of artery calcium and phosphate. The increase in artery calcium and phosphate attributable to warfarin treatment was highly significant (group 3 vs 4; $P<0.001$), as was the decrease in calcium and phosphate attributable to addition of MGP (group 4 vs 5; $P<0.0001$).

The conditioned culture medium from this experiment was examined to determine whether living thoracic aortas secrete MGP, a known inhibitor of the calcification of elastic lamellae in the artery media in vivo.[16,17] As seen in Figure 5, MGP is secreted at a constant rate from 24 to 144 hours in organ culture. The final concentration of MGP in culture medium was 1.5 μg/mL, which is far higher than the 200 ng/mL level of MGP found in the serum of rats of this age. These results show that vascular cells may prevent medial calcification by secretion of calcification inhibitors such as MGP.

Effect of Warfarin and MGP on Serum-Induced Medial Artery Calcification in Living Arteries

Additional experiments were performed to assess the effect of inhibiting the vitamin K–dependent γ-carboxylation of MGP with warfarin, using a dose of warfarin shown previously to inhibit γ-carboxylation in cell culture.[18] As seen in Figure 6, the addition of warfarin to culture medium caused extensive Alizarin red staining for calcification in the living carotid artery segment, whereas no staining could be detected in living carotid arteries incubated in the same medium without warfarin. Supplemental Figure VI shows that this warfarin-induced calcification is largely associated with the elastic lamellae of the artery media. No calcification could be detected if the living arteries were incubated in culture medium containing warfarin but not serum, which confirms the serum requirement for artery calcification in this system. Assay of the conditioned medium from these experiments showed that each 3 to 4 mm segment of carotid artery secreted 0.05 μg MGP per day; this rate is ≈50-fold lower than that observed for thoracic aorta, a difference that is probably attributable to the size difference between the tissues.

A final experiment was performed to determine the effect of adding purified bovine MGP on warfarin-induced calcification in living arteries. As shown in Figure 6, addition of 100 μg MGP/mL completely prevented warfarin-induced carotid artery calcification; similar inhibition of calcification was also seen at 30 μg MGP/mL (data not shown). As expected, purified bovine MGP also prevented calcification of devitalized arteries in the same medium (data not shown).

Discussion

Evidence That Blood Contains a Causative Agent for Medial Artery Calcification

The present studies demonstrate that devitalized rat arteries calcify rapidly when incubated in serum or heparin plasma at the temperature, carbonate concentration, and pH of blood. Calcification is confined to the elastic lamellae of the artery media regardless of the devitalization procedure used and even when cellular material is intentionally fixed in the vascular wall. These observations confirm and extend the results of previous investigations.[8,9]

The present studies further provide the first biochemical characterization of the molecular basis for the calcification of devitalized arteries in serum. The initial characterization experiments showed that devitalized arteries also calcified rapidly when incubated in DMEM containing as little as 1.5% rat serum but not when incubated in DMEM alone. This result demonstrates that blood contains a potent calcification factor, one sufficiently potent that it can still promote calcification in the elastic lamella of the artery when it is present in DMEM at a 70-fold lower concentration than in serum itself. The subsequent experiments showed that this calcification factor consists of ≥1 protein of 50- to 150-kDa molecular weight. The microscopic pattern of serum-induced calcification consists of numerous calcification foci that are scattered within the elastic lamella of the aorta media. These foci are distributed more or less evenly among the different elastic lamellae in sections near the aorta ends (Figure 3) but are typically more numerous in the elastic lamellae nearest the exterior surface of sections in the middle of the 3-cm-long aorta (Figures 2 and 4). This pattern of calcification suggests that diffusion of the nucleating agent from serum into the medial wall may determine the order with which elastic lamellae calcify. All elastic lamellae eventually calcify when devitalized aortas are incubated in serum-containing medium for a prolonged period (supplemental Figure V).

If devitalized arteries calcify so rapidly when incubated in serum, why don't all animals have hardened arteries? The answer to this important question probably lies in the ability of the cells in the artery wall to prevent the serum-initiated calcification of the adjacent elastic lamella by secretion of calcification inhibitors. This hypothesis is supported by the

present observation that thoracic aortas in which cells remain viable during incubation in DMEM containing 15% FCS do not calcify, whereas devitalized aortas calcify rapidly in the same medium (Figure 5). This hypothesis is also supported by the observations that living arteries in organ culture secrete 2 well-established inhibitors of calcification, pyrophosphate,[19] and MGP (Figure 5).

Previous studies have shown that impaired MGP activity causes calcification of the elastic lamellae of the artery media in vivo,[16,17,20] a pattern of calcification essentially identical to that observed in devitalized arteries that have been incubated in serum. It is therefore possible that the MGP secreted by vascular cells in the artery wall contributes to the ability of living arteries to resist serum-initiated calcification in cell culture. Several experiments were performed to test this hypothesis. Inactivation of MGP γ-carboxylation by treatment with warfarin, a treatment known to inactivate the calcification inhibitory activity of MGP in vivo,[17] caused extensive calcification of living arteries in organ culture (Figure 6), and this calcification is restricted to the elastic lamellae of the artery media (supplemental Figure VI). Addition of purified bovine MGP to culture medium completely prevented this warfarin-induced calcification of living arteries in organ culture. These experiments show that MGP secreted by cells in the vascular wall plays an important role in preventing serum-initiated calcification of the elastic lamellae during the organ culture of living arteries and therefore support the hypothesis that the unique susceptibility of the elastic lamellae to serum-initiated calcification in vivo is actively opposed by the secretion of MGP and other calcification inhibitors.

Is Serum Always Required for Calcification Under Cell Culture Conditions?

A discussion of this important question is available in the online supplement.

Possible Relevance of the Calcification of Devitalized Arteries in Serum to Artery Calcification in Chronic Kidney Disease

The present studies show that human serum also has the ability to rapidly calcify devitalized rat aortas, and that the microscopic pattern of this calcification is identical to that seen in aortas incubated in rat serum. However, the calcification of aortas during incubation in human serum appears to be dependent on the phosphate content of the serum sample, and normal adult human serum ([Pi]=1 to 1.5 mmol/L) does not induce artery calcification unless phosphate levels are boosted to ≥2 mmol/L (supplemental Figure VII). A similar dependence of calcification on medium phosphate concentration has also been noted in vascular smooth muscle cells cultured in DMEM with 15% FBS, with no calcification when [Pi]=1.4 mmol/L and significant calcification when [Pi]=2 mmol/L.[21] It is of interest to note that newborn human serum, with [Pi]=3 to 4 mmol/L, induces rapid and specific calcification of the elastic lamella of devitalized aortas without need for phosphate supplementation (personal observations). A discussion of the possible relevance of these observations to the well-established correlation of serum phosphate and artery calcification in chronic kidney disease is available in the online supplement.

Relationship Between the Serum-Initiated Calcification of Arteries and the Serum-Initiated Recalcification of Demineralized Bone

The serum-initiated calcification of arteries is similar to the previously demonstrated serum-initiated recalcification of demineralized bone.[10] These similarities include: (1) a similar time course of calcification when the respective matrices are incubated in neat rat serum at 37°C; (2) a similar dose dilution of the serum activity, with calcification of both matrices occurring in DMEM containing as little as 1.5% serum; (3) a similar molecular weight range of the serum activity, estimated to be ≈50 to 150 kDa in each case; (4) a similar sensitivity of the serum activity to trypsin and chymotrypsin digestion; (5) a similar requirement for the phosphate level needed for calcification in human serum, with no calcification at the 1.2 mmol/L phosphate level found in adult human serum and extensive calcification when the phosphate level is boosted to ≥2 mmol/L; (6) a similar histological appearance of the initial calcification, with numerous calcification foci that could represent discrete nucleation events; and (7) a similar apatite-like mineral phase. Together, these observations strongly suggest that the same calcification factor may be responsible for calcification of collagen and elastin fibers during incubation in serum.

One could well ask why serum might contain a calcification factor. Our working hypothesis is that this calcification factor arises from sites of mineralization in the skeleton, where it is involved in the normal mineralization of the collagenous bone matrix. Because of the vascular nature of bone mineralization sites such as the basic multicellular unit[22]and the bone remodeling compartment,[23] any macromolecule secreted by osteoblasts for the purposes of local bone mineralization will unavoidably escape to blood, just as any serum protein with affinity for mineralizing sites will accumulate in bone. Bone-derived alkaline phosphatase is accordingly released from osteoblasts and appears in serum, and the serum protein fetuin is secreted by hepatocytes and accumulates in bone to become one of the most abundant noncollagenous bone proteins. In our view, the escape of a calcification factor to serum may therefore be just the unavoidable consequence of bone anatomy.

Acknowledgments

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