Matrix Metalloproteinase Inhibition Attenuates Aortic Calcification

Xiao Qin, Matthew A. Corriere, Lynn M. Matrisian, Raul J. Guzman

Objective—Arterial calcification has been associated with matrix metalloproteinase (MMP)–mediated elastin degradation. In this study, we investigated whether inhibiting MMP activity could reduce calcium accumulation in rodent models of aortic calcification.

Methods and Results—Aortic calcification was first induced in male Sprague-Dawley rats by administration of vitamin D₃. Treatment with doxycycline decreased aortic calcium and phosphorus accumulation, and it reduced aortic gelatinase levels; however, it also prevented the bone resorption associated with high doses of vitamin D₃. Using an in vivo model of localized aortic calcification, systemic doxycycline treatment reduced aortic calcium accumulation without affecting serum calcium levels, suggesting a more specific effect of doxycycline in the arterial wall. In organ culture, doxycycline limited aortic calcification caused by exposure to alkaline phosphatase and inorganic phosphate. When GM6001, a synthetic and specific inhibitor of MMPs, was used instead of doxycycline, it had a similar effect. In vivo, periadventitial delivery of GM6001 to calcifying arteries significantly reduced calcification compared with controls.

Conclusions—These results suggest that MMPs are involved in aortic calcification, and inhibiting MMP activity could reduce calcium accumulation in the arterial wall. (Arterioscler Thromb Vasc Biol. 2006;26:1510-1516.)

Key Words: artery ■ calcification ■ MMPs ■ doxycycline ■ GM6001 ■ rat

Vascular calcification occurs pathologically in diabetes and chronic renal disease as well as during the normal aging process.¹–³ When calcification is identified in the coronary arteries, it is associated with an increased risk of cardiac events.⁴,⁵ In patients with renal failure, it is associated with significantly shorter survival,⁶ and in patients with diabetes, it occurs at an accelerated rate and is a strong predictor of morbidity and mortality.⁷ Although vascular calcification is associated with atherosclerosis, recent data suggest that it is independently regulated, and calcifying vessels share features with osteogenesis.²,⁶ Consistent with this hypothesis is that diseased human arteries have been shown to express proteins usually seen in bone, including members of the bone morphogenetic protein family, noncollagenous bone matrix proteins, matrix Gla protein, decorin, the osteoblastic regulator osteoprotegrin, and the matrix metalloproteinases (MMPs).⁹–¹⁵

MMPs are involved in multiple processes in the vascular wall.¹⁶ They are upregulated in human atherosclerotic and restenotic lesions, and they are involved in aneurysm formation.¹⁷–²¹ Inhibiting MMP activity prevents injury-induced arterial remodeling in rodent models.²²,²³ Recent studies demonstrated a correlation between MMP-mediated elastin degradation and aortic calcification. Inhibiting elastin degradation with aluminum ions prevented calcification in the aortas of rats after calcium chloride–mediated injury; and mice deficient in MMP-2 and MMP-9 did not develop calcification in a similar model.²⁴ Additionally, Lee et al demonstrated increased MMP activity associated with increased levels of soluble elastin peptides in a rat subdermal model of elastin calcification.²⁵ In the present study, we sought to determine whether inhibiting MMP activity could prevent calcium accumulation in experimental models of aortic calcification. We demonstrate that doxycycline, an antibiotic that inhibits MMP activity and reduces MMP levels, and GM6001, a synthetic selective MMP inhibitor, can inhibit arterial calcification both in organ culture and in vivo.

Materials and Methods

A detailed Methods section is available in the online supplement, available at http://atvb.ahajournals.org.

Experimental Procedures

Arterial calcification was induced in male Sprague-Dawley rats by subcutaneous injection of 7.5 mg/mL vitamin D₃ for 3 consecutive days. Rats received daily doxycycline by subcutaneous injection beginning at 2 days before vitamin D₃ injections and ending at the time of harvest. In another group of rats, aortic calcification was induced by periadventitial application of 0.15 mol/L CaCl₂ as described recently.²⁴
Calcium, Phosphorus, Bone Densitometry Measurements
Detailed methods for calcium, phosphorus, and bone densitometry measurements are available in the online supplement.

Organ Culture
Segments of rat aorta measuring 1 cm underwent calcification as described previously. Doxycycline (100 μg/mL) was pre-equilibrated in medium and then added to the culture medium. In a separate series of experiments, the synthetic MMP inhibitor GM6001 (12.5 or 25 μmol/L) or GM6001-negative control was used instead of doxycycline.

In Vivo Delivery of GM6001
For in vivo evaluation of GM6001, rats underwent periadventitial application of CaCl₂ solution or normal saline. The synthetic MMP inhibitor GM6001 or GM6001-negative control was then administered via the catheter on a daily basis until the time of aortic harvest.

Results

Doxycycline Inhibits Vitamin D₃-Induced Aortic Calcification In Vivo
Male Sprague-Dawley rats (n=44) were divided into 6 groups that received 3 injections of 7.5 mg/kg vitamin D₃ or vehicle and either 0, 30, 60, or 120 mg/kg doxycycline by daily subcutaneous injection. Histological evaluation of treated and control specimens by von Kossa staining demonstrated extensive medial destruction and calcium staining in vitamin D₃-injected specimens, but this was decreased with increasing dosages of doxycycline. Additionally, at the highest dose of doxycycline used, there was preservation of the elastin structure and medial vascular morphology (supplemental Figure I, available online at http://atvb.ahajournals.org). After 12 days, aortas from animals injected with vitamin D₃ had increased aortic calcification compared with control animals (0.29±0.13 μmol/L/mg calcium in control versus 9.54±1.15 μmol/L/mg in vitamin D₃-injected rats; P<0.05). In vitamin D₃-injected animals that received increasing amounts of doxycycline, there was a dose-dependent decrease in aortic calcium content. Aortic calcium content was 9.54±1.15 μmol/L/mg for 0 mg/kg doxycycline, 5.12±0.28 for 30 mg/kg doxycycline, 3.11±1.12 for 60 mg/kg doxycycline, and 1.49±0.63 for 120 mg/kg doxycycline (ANOVA, P<0.05; vitamin D₃ alone compared with vitamin D₃ plus doxycycline groups, P<0.05; Figure 1). This was mirrored by a dose-dependent decrease in aortic phosphorus content from 0.10±0.01 μmol/L/mg in rats receiving vitamin D₃ to 0.039±0.001 for 30 mg/kg doxycycline, 0.022±0.003 for 60 mg/kg doxycycline, and 0.021±0.004 for 120 mg/kg doxycycline. The average weight change in vitamin D₃-injected animals was different from rats that did not receive vitamin D₃ (−16±9.3 g for vitamin D₃-injected versus 120±13.6 g for control animals; P<0.05), and this weight change was not affected by doxycycline treatment (−22±5.0 g for vitamin D₃ plus doxycycline; P=NS).

Effect of Doxycycline on Vitamin D₃-Induced Aortic MMP Expression, Serum Calcium, and Bone Mineral Density
In rats given vitamin D₃, we observed a decrease in aortic gelatinase levels from rats treated with 60 mg/kg doxycycline compared with those treated with vehicle (Figure 2A). The band corresponded to the band known to be MMP-9 found in conditioned medium from the human HT 1080 cell line (supplemental Figure II). No consistent changes were observed in bands located where MMP-2 is usually seen.

We then evaluated the changes in serum calcium concentration caused by vitamin D₃ injection and 60 mg/kg doxycycline...
cycline treatment. Serum calcium was significantly increased in vitamin D₃-injected rats compared with controls, and this was not affected by treatment with doxycycline. The calcium concentration was 10.58±0.54 mg/dL in control rats versus 15.23±0.44 in vitamin D₃-injected rats (P<0.05 versus controls) and 15.95±0.84 in vitamin D₃-injected plus doxycycline (P=NS compared with vitamin D₃ alone; Figure 2B).

Bone density was significantly lower in vitamin D₃-injected rats than in controls, but doxycycline treatment partially reversed this decrease. Bone density was 0.120±0.002 g/cm² in controls compared with 0.087±0.002 in vitamin D₃-injected rats (P<0.05 versus control) and 0.101±0.005 in vitamin D₃-injected plus doxycycline rats (P<0.05 versus controls; P<0.05 versus vitamin D₃-injected; Figure 2C). This suggests that some effects of doxycycline on aortic calcification could occur through altering the increased bone resorption caused by high doses of vitamin D₃.

**Doxycycline Inhibits Aortic Calcification Caused by Periadventitial CaCl₂ Administration**

We next evaluated whether the effects of doxycycline could be reproduced in a model of arterial calcification that is not characterized by alterations in bone resorption. We studied the effects of doxycycline treatment in rats that underwent periadventitial administration of 0.15 mol/L CaCl₂. Animals not treated with doxycycline demonstrated medial and adventitial accumulation of calcium by von Kossa staining. However, animals treated with doxycycline had nearly complete inhibition of calcification as assessed by the von Kossa stain and by measurement of aortic calcium in harvested specimens. (Figure 3A and 3B). The aortic calcium content was 0.06±0.015 μmol/L/mg for NaCl control rats (n=3), 0.33±0.06 for CaCl₂ rats not given doxycycline (n=4), and 0.13±0.11 μmoles/mg for rats treated with 60 mg/kg doxycycline (n=5; P<0.05; Figure 3C). Additionally, immuno-histochemical staining of these specimens using the macrophage-specific antibody ED-1 demonstrated macrophage infiltration in the adventitia of untreated rats, but this was not seen in doxycycline-treated rats (supplemental Figure III). Serum calcium levels in doxycycline-treated rats were not different from control rats (12.0±0.13 for controls versus 11.27±0.35 for doxycycline-treated rats; P=NS).

**In Vitro Effect of Doxycycline and GM6001 on Arterial Calcification**

We next investigated whether the inhibitory effects of doxycycline on in vivo calcification could be reproduced in an organ culture system. This has the benefit of excluding the effects of doxycycline on systemic, circulating factors. Aortas were calcified in an organ culture system by addition of calf intestinal alkaline phosphatase and sodium phosphate to DMEM without serum (calcification medium).²⁶ von Kossa-stained sections from rat that underwent periadventitial application of calcium chloride and either no doxycycline (−doxy; A) or doxycycline (+: doxy; B). Arrows indicate medial calcium; m; medium; a, adventitia. C, Calcium in aortas from rats that underwent application of NaCl (n=3), CaCl₂ (n=4), and CaCl₂ rats treated with 60 mg/kg doxycycline (n=5). *P<0.05 compared with NaCl control rats; **P<0.05 compared with CaCl₂ rats not given doxycycline.

Calcification medium. Arteries cultured in calcification medium with 100 μg/mL doxycycline demonstrated a significant decrease in calcium accumulation (0.365±0.028; P<0.05 compared with calcification medium alone; Figure 4A). Gelatinase levels in the medium of doxycycline-treated samples were also reduced (Figure 4B).

Although doxycycline has been useful for evaluating a drug with MMP-inhibiting properties, its experimental use is limited because it has several biological effects that are not related to MMPs. To determine whether the effects of doxycycline could be reproduced using a compound with more specific anti-MMP activity, we used the synthetic MMP inhibitor GM6001. Histological evaluation of these samples by von Kossa’s stain failed to demonstrate calcium accumulation in GM6001-treated arteries (Figure 5A and 5B). Aortas exposed to calcification medium showed an increase in calcium content compared with control vessels (0.211±0.016 μmol/L for control compared with 2.525±0.670 for calcification medium). When GM6001 was added to the calcification medium, there was a dose-dependent reduction in aortic calcium accumulation. Aortic calcium was 2.331±0.456 μmol/L for 12.5 μmol/L (P=NS) and 0.434±0.088 for 25 μmol/L GM6001 (P<0.05 versus no GM6001; Figure 5C). Viability of cultured segments at 7
days was not affected by calcification medium or GM6001, as evidenced by measurement of lactic dehydrogenase secretion into the medium, which was not different from control segments (data not shown).

GM6001 Inhibits Aortic Calcification In Vivo

Based on our findings in organ culture, we next evaluated whether GM6001 could be used to inhibit aortic calcification in vivo. Daily injections of GM6001 or its negative control (250 μg per day) were administered by catheter into the periaortic space after periadventitial application of calcium chloride. Aortas from control rats that underwent painting with NaCl had minimal calcification after 7 days. However, CaCl₂-painted arteries treated with GM6001-negative control had a >10-fold increase in calcium accumulation, and this was significantly decreased by local delivery of GM6001 (0.166 ± 0.026 μmol/L for normal saline controls; 1.983 ± 0.477 for CaCl₂ with GM6001-negative control; and 0.656 ± 0.331 for CaCl₂ painted vessels treated with GM6001; P < 0.05). Aortic histology from rats that received the negative control compound demonstrated extensive calcification in the adventitia and in the outer medial layers, whereas there was only occasional calcium staining in aortas from rats treated with GM6001 (Figure 6).

Discussion

In this study, we found that inhibiting MMP activity is associated with decreased calcium accumulation in the arterial wall. We began our studies by using doxycycline, an antibiotic with known MMP-inhibiting properties, to demonstrate an association between decreased aortic gelatinase levels and reduced aortic calcification. To explore the importance of MMPs in arterial calcification more directly, we used the synthetic MMP-specific inhibitor GM6001 and were able to demonstrate that it decreased aortic calcification both in vitro and in vivo. These findings suggest that MMPs play an important role in arterial calcification, and further, that inhibiting MMP activity may prevent arterial calcification in the clinical setting.

The changes in arterial histology associated with hypervitaminosis D have been well characterized. Rats exposed to sublethal doses of vitamin D initially develop calcium phosphate crystals along the elastic laminae followed by spreading of calcification into the surrounding medial smooth muscle cells. The model is characterized by increased bone resorption and increased intestinal uptake of ingested calcium, leading to elevated serum calcium levels. Although the mechanisms related to arterial calcification in this model have not been fully elucidated, it is thought to occur through an interaction between degraded medial elastin and circulating factors from resorbed bone. Price et al demonstrated recently that a fetuin-matrix Gla protein–mineral complex is increased in the circulation of rats exposed to toxic doses of
vitamin D, whereas serum levels of fetuin, a proposed inhibitor of calcification, are decreased. They propose that high levels of fetuin in complex may prevent it from inhibiting the growth of mineral components in the elastic layer of the vessel wall. Alternatively, the fetuin–mineral complex might serve as a marker for a presently unknown causative factor.

In our experiments, administration of vitamin D, increased MMP-9 levels in the aortic wall and increased serum calcium levels. Doxycycline treatment reduced aortic MMP-9 levels back to normal, but it had no effect on serum calcium levels. This suggests that doxycycline may act in a manner that is similar to ibandronate or osteoprotegrin, which inhibit bone resorption but do not affect serum calcium levels in vitamin D-treated rats. However, we have not excluded the possibility that the effects of doxycycline are related to other mechanisms such as increases in inhibitory factors, decreases in positive regulatory factors, direct binding of doxycycline to hydroxapatite, antibiotic properties, or other yet unknown factors. Additionally, there are known interactions between doxycycline and calcium, and these may have occurred in our model despite the lack of effect on serum calcium levels. Also notable is the significant weight loss seen in vitamin D-treated rats. This weight loss was not reversed by treatment with doxycycline, which suggests that vitamin D administration might have indirect consequences. For example, leptin levels may have been affected in this model, and this may have resulted in indirect effects on vascular calcification.

The importance of MMPs in arterial calcification has been suggested previously by Basalyga et al, who demonstrated that mice deficient in MMP-2 or MMP-9 failed to develop aortic calcification after perivascular application of CaCl₂. Additionally, Lee et al recently demonstrated by gelatin zymography and RT-PCR that MMP-2 and MMP-9 levels are increased in subdermally placed elastin that is undergoing active calcification. In the present series of experiments, we demonstrated that MMP-9 levels are increased in calcifying aortas, and that decreased gelatinase levels are associated with a reduced amount of aortic calcium accumulation. However, other MMPs are also capable of degrading medial elastin, and it is possible that MMPs other than the gelatinases could be responsible for the elastin disruption seen in this model.

A mechanism for elastin calcification that involves a positive feedback loop between degraded elastin and MMP gene expression has been proposed recently. According to this model, physical or biochemical injury may disrupt the glycoproteins normally surrounding elastin. This may serve to expose elastin to cells that, in turn, produce MMPs or other serine proteases that can degrade elastin. MMPs can then further degrade both the protective glycoproteins in the arterial wall and the exposed elastin. Elastin degradation may also release matrix-bound cytokines that can recruit inflammatory cells and cause smooth muscle cells to undergo a phenotypic modulation into more osteoblastic-type cells. Our data on aortic gelatinase levels during calcification are consistent with this model and furthermore suggest that the enzymes required for elastin degradation can be generated within the arterial wall.

A recently described model of aortic calcification involves the perivadventitial application of CaCl₂. Because calcification in this model is related to local factors, it is not associated with altered circulating levels of calcium, phosphate, or other products of bone resorption. Using this model, we were also able to demonstrate a significant reduction in aortic calcium accumulation by systemic treatment with doxycycline. The inhibitory effects of doxycycline on arterial calcification in this model argue against a mechanism related specifically to circulating mineral complexes; however, there are ongoing low levels of bone resorption that may be inhibited by doxycycline, and even these minimal changes may be sufficient to affect arterial calcification. Additionally, we noted strong ED-1–specific staining in the adventitia of calcifying aortas, but this was not seen in doxycycline-treated rats.

Figure 6. The MMP inhibitor GM6001 inhibits aortic calcification in vivo. Rats were subjected to periadventitial application of CaCl₂ or normal saline, and then they were treated with either GM6001 or its negative control compound (neg-cont). A and B, von Kossa staining shows calcium in aortas from rats that did not receive GM6001 (−GM6001; A) and in aortas from rats that received local treatment with GM6001 (+GM6001; B). Arrows indicate calcium; m, medium; a, adventitia. C, Aortic calcium concentration in saline controls (n = 6), CaCl₂-painted rats that received GM6001-negative control (n = 11), and CaCl₂-painted vessels that received GM6001 (n = 9); ANOVA *P < 0.05; **P < 0.05 compared with saline control; ***P < 0.05 compared with rats receiving negative-control compound.
Although the role of macrophages in this model has not been defined, it is possible that they are another source of matrix-degrading enzymes necessary for the calcification to proceed. The potential role of inflammatory cells and their secreted mediators on arterial calcification has been reviewed recently, and further studies in this regard are needed.

Our experiments in organ culture allowed us to focus on the direct effects of MMP inhibition on the aortic wall. In this system, calcification is thought to occur because of inhibition of pyrophosphate production and increased calcium-phosphorus product in the medium. Doxycycline reduced aortic calcium levels, and this effect was associated with decreased MMP levels in the culture medium. This likely occurred through the previously described inhibitory effects of doxycycline on MMP synthesis. However, the use of doxycycline in mechanistic studies is limited because of its diverse actions, many of which are not related to altering MMP levels. For this reason, we extended our experiments to include the synthetic compound GM6001 that specifically inhibits the MMPs. We found that addition of GM6001 to the calcification medium resulted in a dose-dependent decrease in arterial calcium accumulation. On histology, GM6001-treated vessels had less calcium deposition in the adventitia and outer medial layers. GM6001 is a potent, hydroxamic acid–based MMP inhibitor with activity against several MMPs, including collagenase, the gelatinases, and stromelysin. Both the gelatinases and stromelysin have elastolytic activity, and a possible role of MMPs in medial calcification is in initiating the process of elastin degradation that may serve to provide a nidus for hydroxyapatite crystals to develop. These in vitro results suggested that synthetic MMP inhibitors might prevent arterial calcification in vivo.

Our final series of experiments involved the use of GM6001 in vivo. For these studies, GM6001 was delivered locally, near the aorta at a concentration that was similar to that used in organ culture, and we found that daily administration reduced aortic calcium content compared with negative control treated vessels. Although the source of MMP activity in this model is presently unknown, the potential sources include medial smooth muscle cells, adventitial fibroblasts, or infiltrating inflammatory cells, all of which would have been affected by local delivery of the inhibitor. GM6001 is a synthetic inhibitor with activity against several MMPs, and thus, the identity of MMPs involved in arterial calcification remains to be determined. Based on the current model, it is likely to be an MMP with elastolytic properties that can be produced either within the aortic wall or by cells that can reside in the adventitia. One possibility is that GM6001 has direct effects on the CaCl2 solution used to inhibit calcification. However, the accumulation of aortic calcium we observed with the negative control compound argues against this point. Another limitation of this technique is that compound delivery is limited to 5 to 7 days because of the formation of a fibrous capsule around the catheter. However, the fact that catheter delivery was successful suggests that aortic calcification occurs early in this model and that it can be inhibited by drug administration during the first several days. Alternatively, sufficient compound may have diffused through the capsule to have an effect at later time points. Efforts to understand the temporal aspects of this model and the relative contributions of MMPs during the various phases of arterial calcification are under way.

Together, our data suggest that MMPs are involved in arterial calcification and that inhibiting MMPs may be a clinically useful method for reducing calcification in the vessel wall. Arterial calcification is a complex phenomenon that is likely regulated at several levels, and further efforts to understand the role of MMPs in this pathologic process are needed.

Acknowledgment

We thank Dr Richard Hoover for his support and assistance in producing this manuscript.

Sources of Funding

This research was supported by mentored clinical scientist development award HL069926 from the National Heart, Lung, and Blood Institute (R.G.) and grants from the Lifeline Foundation and the William J. von Liebig Foundation.

Disclosures

None.

References


Matrix Metalloproteinase Inhibition Attenuates Aortic Calcification
Xiao Qin, Matthew A. Corriere, Lynn M. Matrisian and Raul J. Guzman

Arterioscler Thromb Vasc Biol. 2006;26:1510-1516; originally published online May 11, 2006;
doi: 10.1161/01.ATV.0000225807.76419.a7

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/26/7/1510

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/05/21/01.ATV.0000225807.76419.a7.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org/subscriptions/
MATERIALS AND METHODS

Materials

Vitamin D₃ (cholecalciferol) and doxycycline hyclate were purchased from Sigma-Aldrich Chemical Co., Inc. (St. Louis, MO). The synthetic MMP inhibitor GM6001 and its negative control GM6001-negative-control were purchased from Calbiochem, EMD Biosciences, Inc, an Affiliate of Merck KGaA, (Darmstadt, Germany). Vitamin D₃ solution for injections was prepared as described by Price.¹ A stock solution was made by dissolving 0.033g vitamin D₃ in 200µl ethanol and suspended in 1.4ml of Emulphor (alkamuls EL-620) before diluting it to 1.65 mg/ml (66,000IU/ml) in 20 ml D₃W. Doxycycline hyclate was dissolved in water to give a concentration of 20 mg/ml. Male Sprague-Dawley rats weighing 100 g were purchased from Charles River (Bedford, MA).

Experimental Procedures

All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. Animals were housed in accordance with institutional policies and fed, ad libitum with Purina 5001 rodent chow containing 0.67% phosphorus and 0.95% calcium by weight. In order to induce aortic calcification, rats were given subcutaneous injections of 7.5 mg/ml Vitamin D₃ for three consecutive days. For evaluation of its effects on aortic calcification, rats received 0, 30, 60, or 120 mg/kg doxycycline daily by subcutaneous injection beginning at 2 days prior to vitamin D₃ injections and ending at the time of harvest. Rats were weighed prior to the experiment and immediately before vessel harvest. Animals that appeared to be in distress were euthanized and not used for analysis. Mortality was less than 5% in each group. On the day of aortic harvest,
animals were overdosed with pentobarbital and the aorta was harvested from the arch to the iliac bifurcation. Blood was collected from the inferior vena cava and the serum was collected and stored at -70°C until needed for further evaluation. In order to insure consistency, the thoracic aorta was used routinely for histologic evaluation, the infra-renal aorta for gelatin zymography, and a 1 cm portion of aorta at the level of the mesenteric vessels for evaluation of calcium and phosphorus levels.

In another group of rats, aortic calcification was induced by periadventitial application of 0.15 M CaCl₂ as recently described.² Anesthesia was induced using Enflurane and rats were anesthetized using 1.5 mg/kg ketamine and 15 mg/kg xylazine. Through a midline incision, the aorta was exposed and CaCl₂ or 0.9% NaCl as a control were applied with 15 paint strokes using a cotton tipped swab. Two days prior to CaCl₂ application, animals began treatment with 60 mg/kg of doxycycline or water and this was performed daily until aortic harvest. One week after periadventitial CaCl₂ administration, animals were anesthetized with ketamine and xylazine then euthanized by exsanguination. A segment of infrarenal aorta was excised and processed for calcium determination and histology.

**Calcium and Phosphorus measurements**

Samples of aorta measuring 1 cm were incubated in 150 mM HCl for 24 hours with gentle agitation. Calcium content was measured using a modified o-cresolphthalein complexone method;³ and phosphorous content was measured using the ammonium molybdate method.⁴ Specimens were then dried and weighed. Results are expressed as µmoles calcium per mg of aortic tissue for in vivo experiments and µmoles calcium for
organ culture experiments. Serum calcium was measured by a commercial laboratory (Antech Diagnostics, Southhaven, MS).

**Bone Densitometry measurements**

Bone density was measured 6 days after beginning vitamin D₃ treatment. Rats were anesthetized with ketamine and xylazine then imaged using the GE PIXImus™ bone densitometry system (Wipro GE Healthcare) which uses DEXA (dual-energy x-ray absorptiometry) to determine bone mineral density. Equal size regions of interest were selected manually to include the left femoral shaft, and bone density, measured as g mineral/cm², was determined automatically using the system software.

**Histology and Immunohistochemical staining**

For histology, the thoracic aorta was harvested within 10 minutes of death and rinsed in PBS prior to fixation in 4% paraformaldehyde for 30 minutes. Specimens were then dehydrated and embedded in paraffin for sectioning. Sections were stained by hematoxylin and eosin and serial sections were stained using the von Kossa method (5% AgNO₃ for 1 hour under light) and counterstained with hematoxylin. For immunohistochemistry, sections were hydrated, incubated in blocking serum, and then incubated with the macrophage specific antibody ED-1 (Research Diagnostics, Inc., Flanders, NJ) overnight at 4°C. Vessels were then incubated with secondary anti-mouse antibody followed by DAB peroxidase substrate (Vector Laboratories, Inc, Burlingame, CA) and then counterstained with hematoxylin.

**MMP Gel zymography**

Sections from the abdominal aorta of vitamin D₃-injected rats were harvested at various times and then snap frozen in liquid nitrogen until needed. Protein from aortic specimens
was extracted in a sodium dodecyl sulfate (SDS) containing buffer and then equal amounts of protein from each extract were electrophoresed on an 8% SDS–polyacrylamide gel containing 0.1% type I gelatin (J.T. Baker, Phillipsburg, NJ). Gels were washed with 2.5% Triton X-100, incubated overnight in 0.05 mol/L Tris with 2.5 mmol/L CaCl$_2$ and 0.02% NaN$_3$, stained with Coomassie blue, then destained in methanol and acetic acid solution. Gelatin degradation was observed as white lytic bands. Gels were digitally photographed and bands were quantified using the Bio-Rad Quantity One software. Conditioned medium from the HT1080 human fibrosarcoma cell line was used to mark MMPs in the Zymograms.

**Organ Culture**

Segments of rat aorta measuring 1 cm were harvested and gently cleared of surrounding tissues. Aortic segments underwent calcification as previously described.$^5$ Briefly, aortic segments were incubated in DMEM (Gibco, Long Island, NY) containing 1X penicillin/streptomycin and without serum at 37°C in a humidified 5% CO$_2$ / 95% air atmosphere. In order to induce calcification, 7.5 units/ml of calf intestinal alkaline phosphatase was added to DMEM and the phosphate ion concentration was increased to 3.8 µM by addition of sodium phosphate (calcification medium). Doxycycline (100 µg/ml) was pre-equilibrated in medium and then added to the culture medium. In a separate series of experiments, the synthetic MMP inhibitor GM6001 (12.5 or 25 µM) or GM6001 negative control were used instead of doxycycline. Culture medium with additives was pre-incubated in order to allow it to equilibrate at 37°C and pH=7.4 before applying to specimens. After 9 days, aortic specimens were rinsed in normal saline prior
to decalcifying in 500 µl of 0.15 M HCl for 24 hours with gentle agitation. Calcium was then measured in 50 µl samples of the HCl solution as described above.

**In vivo delivery of GM6001**

For in vivo evaluation of GM6001, rats initially underwent peri-adventitial application of CaCl₂ solution or normal saline to induce calcification as described above. Immediately after CaCl₂ administration, PE-50 tubing with an occluded end and multiple side perforations was placed adjacent to the infrarenal aorta. The other end of the catheter was the tunneled subcutaneously to the intrascapular region and exteriorized. The synthetic MMP inhibitor GM6001 was prepared by dissolving 5 mg of powdered compound in 1 ml DMSO. For each dose, 25 µl of compound in DMSO was diluted to 400 µl in 0.9% NaCl. The commercially supplied negative control, named GM6001-negative-control, was prepared in an identical manner. Compounds were administered via the catheter on a daily basis until the time of aortic harvest. After 7 days of treatment, the aorta was harvested and processed for calcium accumulation and histology as described above.

**Cell viability assay**

For organ culture studies, cellular viability of aortas was assessed at 3 days and at 7 days using the CytoTox-ONE homogenous membrane integrity assay (Promega Corp, Madison, WI) which measures the release of lactate dehydrogenase from cells. The assay was conducted using 100 µl of conditioned medium and according to the supplied protocol.
Statistics

Data are presented as mean ± standard error of the mean. For in vivo studies, data was initially evaluated using analysis of variance (ANOVA) followed by post-hoc evaluation using Bonferroni’s method with p<0.05 considered significant. For organ culture studies, group comparisons were performed using the Kruskal-Wallis test followed by pair wise comparisons using the Mann-Whitney U test.

REFERENCES


**Figure I.** Representative sections of von Kossa stained aortic specimens show a normal vessel (A), followed by aorta from vitamin D$_3$-injected rat that did not receive doxycycline (B), aorta from Vitamin D$_3$-injected rats that received 30 mg/kg (C), 60 mg/kg (D), and 120 mg/kg (E) doxycycline by injection. Arrows denote calcium staining, m = media, a = adventitia.
**Figure II.** A. Gelatin zymography of aortic lysates from vehicle treated controls, vitamin D₃-injected and vitamin D₃-injected plus 60mg/kg doxycycline treated rats.

**Figure III.** Immunohistochemistry for macrophages using ED-1 antibody. Aortas from control rats that underwent aortic painting with CaCl₂ and received vehicle alone (A, B) or 60 mg/kg doxycycline (C). Large arrows denote areas of calcification, small arrows denote areas of positive ED-1 staining, m = media, a = adventitia
**Figure IV.** Von Kossa staining of organ culture vessels incubated in calcification medium (A), or in calcification medium with doxycycline (B). Arrows denote calcium in arterial media.