Inhibition of Matrix Metalloproteinase Activity Attenuates Tenascin-C Production and Calcification of Implanted Purified Elastin in Rats

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Elastin, a major extracellular matrix protein present in arterial walls provides elastic recoil and resilience to arteries. Elastin is prone to calcification in a number of cardiovascular diseases including atherosclerosis and bioprosthetic heart valve mineralization. We have recently shown that purified elastin when implanted subdermally in rats undergoes severe calcification. In the present study, we used this elastin implant model to investigate the molecular mechanisms underlying elastin calcification. Intense matrix metalloproteinase (MMP-2) and tenascin-C (TN-C) expression were seen in the proximity of the initial calcific deposits at 7 days. Gelatin zymography studies showed both MMP-2 (latent and active form) and MMP-9 expression within the implants. To investigate the role of MMPs in calcification, rats were administered a MMP inhibitor, (2S-allyl-N-hydroxy-3R-isobutyl-N-(1S-methylcarbamoyl-2-phenylethyl)-succinamide (BB-1101) by daily injection, either systemically or at the implant site. The site-specific BB-1101 administration almost completely suppressed TN-C expression, as shown by immunohistochemical staining, within the implants. The systemic BB-1101 injections also significantly reduced TN-C expression within the elastin implants. Moreover, calcification of elastin implants was significantly reduced in the site-specific administration group ($5.43 \pm 1.03 \mu\text{g}/\text{mg Ca}$ for BB-1101 group *versus* 21.71 ± 1.19 for control group, $P < 0.001$). Alizarin Red staining clearly showed that the elastin fibers were heavily calcified in the control group, whereas in BB-1101 group the calcification was scarce with few fibers showing initial calcification deposits. The systemic administration of BB-1101 also significantly reduced elastin calcification (28.07 ± 5.81 control *versus* 16.92 ± 2.56 in the BB-1101 group, $P < 0.05$), although less than the site-specific administration. Thus, the present studies indicate that MMPs and

TN-C play a role in elastin-oriented calcification. (Am J Pathol 2000, 157:885–893)

Elastin is an extracellular matrix protein present in a variety of tissues including the arterial wall and heart valves.¹ Pathological calcification of elastin occurs in a number of disease processes including atherosclerosis, cardiac valve disease, and bioprosthetic heart valve calcification.^{2–4} Despite the importance of elastin calcification in cardiovascular disease, the mechanisms underlying this process are not fully understood. We recently characterized a rat subdermal implant model to study calcification of purified elastin.⁵ Explants from these animals showed deposition of poorly crystalline hydroxyapatite on implanted elastin fibers, comparable to pathological cardiovascular calcification.⁵ This system is therefore useful for determining the cellular and molecular mechanisms leading to elastin-oriented calcification.

Although the elastic fibers can be considered physiologically inert during adult life, a wide range of insults to elastic tissue can result in either chronic loss or excess accumulation.⁶ Matrix metalloproteinases (MMPs) are involved in elastolysis. In particular, both MMP-2 and MMP-9 are known to bind to insoluble elastin,⁷ and each has been shown to be actively involved in elastin degradation.^{8,9} Exuberant production of MMPs is a hallmark of many destructive diseases, such as arthritis, chronic ulceration, and tumor formation.^{10–12} With respect to calcification, MMPs have also been detected in association with calcification of bioprostheses.^{13,14} For example, subdermally implanted glutaraldehyde-treated bovine parietal pericardium contains an array of extracellular matrix protein-degrading proteinases including serine proteinases and MMPs.^{13,14} High concentrations of MMPs are also present in atherosclerotic plaques¹⁵ and in restenotic lesions.¹⁶

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Tenascin-C (TN-C) is an extracellular matrix glycoprotein with a highly restricted pattern of gene expression, but it is prominently expressed in embryonic and adult tissues that are actively remodeling.¹⁷ A number of studies indicate that MMPs regulate TN-C expression.^{18,19} For example, after arterial injury, TN-C and MMPs²⁰ are up-regulated during the development of occlusive neointimal lesions, whereas inhibition of MMP activity attenuates this process.²¹ In addition, both MMP-2²² and TN-C^{23,24} are able to bind the same cell surface receptor, the $\alpha v \beta 3$ integrin, further indicating that their regulation and functions may be interdependent. In fact, we have recently shown that extracellular matrix protein proteolysis by MMPs activates TN-C transcription via an ERK1/2 MAPK-dependent signaling pathway.^{18,25}

With respect to calcification, a number of studies indicate that there is a strong relationship between TN-C expression and calcification in normal and dystrophic mineralization. For example, TN-C is expressed in developing bone²⁶ and co-localizes with the calcium-binding protein S-100 β in the cranium.²⁷ During tooth development, TN-C is expressed by the peridontoblast at the inner enamel mineralization front.²⁸ In addition, tissue culture studies demonstrate that osteoblast adhesion to TN-C up-regulates alkaline phosphatase, a well-established marker of bone differentiation.²⁹ Other studies suggest that TN-C may act as a mediator of TGF- β -dependent bone formation,³⁰ as well as pericyte differentiation/mineralization during neovascularization.³¹ Moreover, physical loading and the resulting increased strain imposed on rat ulnae leads to early increases in osteoblast TN-C expression, indicating that this protein may act as a mediator of osteoregulatory responses to altered biomechanics.³² Despite these studies, the mechanistic and functional links that may exist between MMP and TN-C expression during elastin-oriented calcification *in vivo* have not been examined.

In the present study, we investigated the production and activity of MMPs and TN-C during early (3 and 7 days) elastin implant calcification in rats by immunohistochemistry and gelatin substrate zymography. Furthermore, using a hydroxamate-based MMP inhibitor (BB-1101), we tested the hypothesis that systemic or site-specific inhibition of MMP activity would attenuate elastin calcification. Inhibition of MMP activity not only resulted in a reduction of elastin calcification, but also reduced TN-C production within elastin implants. Moreover, site-specific delivery of BB-1101 was more effective in reducing both TN-C and calcification. These studies indicate that MMPs are important mediators of both TN-C production and elastin-oriented calcification.

Materials and Methods

Elastin (5- to 10-mm fibers) from bovine neck ligament purified by a neutral extraction method was obtained from the Elastin Product Company (Owensville, MO). 2S-allyl-N-hydroxy-3R-isobutyl-N-(1S-methylcarbamoyl-2-phenylethyl)-succinamide (BB-1101) was a kind gift from British Biotech Pharmaceuticals Ltd. (Ox-

ford, UK). Mouse anti-MMP-2 monoclonal antibody (Calbiochem, La Jolla, CA), human TN-C monoclonal antibody (DAKO, Carpinteria, CA), monoclonal mouse anti-rat CD3 antibody (Genzyme Diagnostics, Cambridge, MA), monoclonal mouse anti-rat macrophages (Biosource International, Camarillo, CA), monoclonal mouse anti-human vimentin (Sigma Chemical Co., St. Louis, MO), and monoclonal mouse anti-human α -smooth muscle actin (Sigma) were used in immunohistochemistry studies. An alkaline phosphatase staining kit was obtained from Boehringer-Mannheim (Mannheim, Germany). Ketamine HCl (Ketaset; Fort Dodge Lab, Fort Dodge, IA) and xylazine (Rompun; Miles Inc., Shawnee Mission, KS) were used for rat anesthesia.

Rat Subdermal Implantation

Male Sprague-Dawley rats (21 to 24 days old, 50 to 65 g; Charles River Laboratories, Burlington, MA) were anesthetized by intramuscular injections of ketamine HCl and xylazine (4:3), 0.001 ml/g of body weight. Using a sterile technique, a small incision was made on the back of the rats and two subdermal pouches (one in front and one in back) were created. Two samples of elastin fibers were then implanted subdermally in the pouches. The rats (five per group) were sacrificed by CO₂ asphyxiation at 3 days and 7 days and samples were retrieved. The samples (10 per group) were used for quantitative calcium and phosphorus analyses, and three samples per group were snap-frozen over dry-ice in frozen OCT (tissue embedding medium) and three samples per group were fixed in phosphate-buffered formalin fixative for morphological and immunohistochemical studies. All immunohistochemical-staining studies were performed with at least three different samples in each group.

MMP Inhibitor Studies

BB-1101 (30 mg) was suspended in 0.6 ml of ethanol and then 11.4 ml of phosphate-buffered saline (PBS) was added drop-wise with sonication to obtain a stable suspension. The control solution was made without BB-1101. This suspension was then administered preimplantation to ensure adequate drug levels in rats by subdermal injections (10-mg/kg body weight/day), either at the implant site (the back of the rats) or on the abdominal side (systemic). One day after initial administration of BB-1101, elastin samples were implanted (five rats per group, two samples in each rat) as described above. The drug therapy was continued once per day for 7 days. The drug suspension was sonicated each day before administration. The doses were chosen according to the manufacturer's suggestion. Plasma concentrations of the drug were not determined. Rats were sacrificed by CO₂ asphyxiation at 3 days and 7 days, and elastin samples along with the tissue capsule were retrieved and used for quantitative calcium and phosphorus determination and immunohistochemistry and zymography studies.

Immunostaining for MMPs and TN-C

Explanted elastin samples were fixed overnight in neutral-buffered formalin and embedded in paraffin. Sections (6 μm) were taken for histology, deparaffinized using xylene, and sequentially rehydrated in graded ethanol. Vectastain ABC kit (Vector Laboratories, Burlington, Ontario, Canada) was used for immunoperoxidase staining according to the manufacturer's instructions. Mouse anti-MMP-2 monoclonal antibody and human TN-C monoclonal antibody diluted 1:100 in wash buffer were used for 2 hours at 37°C. Immune complexes were then stained by incubation in a solution of 3, 3'-diaminobenzidine tetrahydrochloride dihydrate, (diaminobenzidine substrate kit, Vector Laboratories), and hydrogen peroxide. Sections were then counterstained with hematoxylin and eosin. For all immunohistochemistry experiments, negative controls included omission of the primary antibody and substitution of nonspecific mouse antisera.

Assessment of Cellular Invasion

Similar immunohistochemical protocols as described above were performed to characterize T cell, fibroblast, pericyte, and macrophage infiltration within the elastin implant using monoclonal antibodies against rat CD3 cells (mouse anti-rat CD3), vimentin (cell marker for fibroblasts), α -smooth muscle actin (for activated fibroblasts, smooth muscle cells and pericytes), and rat macrophages (mouse anti-rat macrophages). Anti-rat CD3 recognizes the rat T cell homologue of human CD3.

Zymography

Explanted elastin samples were homogenized in extraction buffer containing 50 mmol/L Tris, 0.2% Triton X-100, 10 mmol/L CaCl_2 , and 2 mol/L guanidine.HCl, pH 7.5. The extracts were centrifuged at $10,000 \times g$ for 10 minutes and the supernatants were dialyzed overnight against 0.2% Triton X-100, 50 mmol/L Tris, pH 7.5. The protein content of the extract was determined using the biocinchoninic acid method according to the manufacturer's procedure (Pierce Inc., Rockford, IL). Samples containing equal amounts of protein (5 to 20 μg per lane) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel with 0.1% (wt/vol) gelatin (Bio-Rad, Richmond, CA) under nonreducing conditions. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 minutes to remove sodium dodecyl sulfate and then incubated in the substrate buffer at 37°C (50 mmol/L Tris buffer, pH 7.8, containing 10 mmol/L CaCl_2) for 70 hours. The gels were then stained with 0.02% Coomassie brilliant blue (Sigma Chemical Co.). MMPs appear as areas of lysis on a dark blue Coomassie-stained gelatin background. For selected gels, 100 μL BB-1101 (100 $\mu\text{g}/\text{ml}$) was added to the substrate buffer to study the MMP inhibitory effects of BB-1101.

Alkaline Phosphatase Staining

The alkaline-phosphate staining solution was prepared by adding 50 μL 4-nitro blue tetrazolium solution and 37.5 μL X-phosphate solution (50 mg/ml in dimethyl formamide) to 10 ml 0.1 mol/L of Tris buffer (pH 9.5, 0.05 mol/L MgCl_2 , 0.1 mol/L NaCl). The frozen sections were rinsed with the same buffer used for the staining solution. The sections were then incubated in the staining solution in the dark for 1 hour at room temperature. After development, the sections were washed in water, and then were fixed in 4% paraformaldehyde solution for 5 minutes at room temperature. Sections were washed with PBS and mounted with a mounting medium.

Calcification Assessment

Quantitative calcium and phosphorus analyses were performed on the explanted samples as per established procedures.^{33,34} Briefly, explanted elastin samples were lyophilized, weighed, and then digested in 6 N HCl at 95°C for 24 hours in a closed container. The solution was then completely evaporated at 70°C with a continuous flow of nitrogen in the tube. The residue left in the test tube was then dissolved in 1 ml of 0.01 N HCl. The calcium content of each sample was based on the aliquot concentration of each hydrolysate using atomic absorption spectroscopy according to the established procedures.³³ The phosphorus levels were determined by molybdate complexation assay.³⁴ Representative frozen sections of explants were stained with Alizarin Red staining for localization of calcium phosphate deposits.

Data Analyses

Quantitative results were expressed as the means $\pm$ SE. Differences between control means and treated groups were assessed using the unpaired Student's *t*-test. Data were termed significant when $P < 0.05$.

Results

Assessment of Early Calcification

Elastin fibers, when implanted subdermally in rats, underwent progressive calcification as determined by quantitative calcium and phosphorus analysis (Figure 1). At 3 days, calcification was evident at the peripheral elastin fibers and at 7 days, calcification was evident throughout the elastin implant as shown by Alizarin Red staining (Figure 2F). Calcium and phosphorus levels within the elastin implants were increased at 7 days. The molar ratio of calcium to phosphorus, ~ 1.8 , suggested deposition of poorly crystalline hydroxyapatite.

MMP-2 and TN-C Production within Elastin Implants

At 3 days after implantation, host cells began to invade the surface of the elastin implant. This was associated

Figure 1. Rat subdermal calcification data for elastin for the 3- and 7-day time-course study ($n = 10$ per group).

with moderate staining for MMP-2 and low immunoreactivity for TN-C (Figure 2, A and B). At 7 days, extensive cellular infiltration had occurred and this corresponded with intense staining for MMP-2 and TN-C throughout the implant (Figure 2, D and E). IgG-stained controls were negative for MMP-2 and TN-C (Figure 2C). Moreover, the intense MMP-2 and TN-C staining was apparent near the calcific deposits on elastin as seen by Alizarin Red staining at 7 days (Figure 2F).

Characterization of Cellular Infiltrates

At 7 days, elastin implants were encapsulated by host tissue, and were extensively invaded by host cells (Figure 3A). Immunostaining indicated that numerous CD3-pos-

itive T cells were present in the outer capsule and within the implant (Figure 3B). Alpha smooth muscle cell actin antibody recognizes pericytes and activated fibroblasts. This staining showed the presence of pericytes in small blood vessels forming within the capsule and an occasional staining for activated fibroblasts close to elastin fibers (Figure 3C). Vimentin staining for fibroblasts showed heterogeneous staining throughout the capsule with strong staining in close proximity of elastin fibers (Figure 3D). Only occasional cells were stained, if any, with the macrophage antibody suggesting low levels of macrophages surrounding the implant (Figure 3E).

MMP Inhibitor Studies

To determine whether MMPs regulate TN-C and elastin calcification, rats were treated daily with an MMP inhibitor, BB-1101, either locally or at the distal site (systemically) for 7 days. Gelatin substrate zymography was used to assess the type and activity of MMPs in elastin implants. In the control vehicle group, gelatinolytic bands were seen at approximate molecular weights 90, 68, and 57 kd (Figure 4). The band at 90 kd most likely represents MMP-9. Under the denaturing and nonreducing conditions used for zymography, the pro-form of MMP-2 has an apparent molecular weight of 68 kd, whereas the active form of MMP-2 appears at ~57 kd. Thus, our data indicates that both MMP-2 and MMP-9 are present within elastin implants. When BB-1101, an MMP inhibitor was added to the substrate buffer while performing zymography, no lytic bands were seen confirming that the zymography bands seen were because of MMPs, and BB-1101 nonspecifically inhibits all MMP activity (Figure 4). When rats were administered BB-1101, either at the implant site or systemically, the MMP activity within the implant was

Figure 2. Immunohistochemical localization of MMP-2 and TN-C within the elastin implants. At 3 days both MMP-2 and TN-C are expressed at the periphery of the implant (A and B), the control sera was negative (C); at 7 days intense staining was seen for MMP-2 and TN-C throughout the elastin implant (D and E) and Alizarin Red staining shows calcification of elastin fibers (F). Original magnifications, A–F, $\times 10$. EL, elastin implant; CP, surrounding capsule.

Figure 3. Cellular infiltration of elastin implant. At 7 days, the elastin implant was invaded throughout with the host cells (**A**, H&E staining). Numerous T cells (**B**) and fibroblast-like cells (**C** and **D**) were seen in the outer tissue capsule while little to no staining was evident for macrophages (**E**). Original magnifications, **A–E**, $\times 40$. EL, elastin implant; CP, surrounding capsule.

not decreased as shown by immunohistochemistry (data not shown) and zymography (Figure 4), which is expected, as this drug does not act at the expression level. As the BB-1101 chelation is reversible, it is possible that BB-1101 would be removed from elastin explants during

extraction and washing procedures before gelatin zymography.

Next, we looked at the downstream effects of inhibiting MMP activity. In the control group, TN-C-positive staining was seen throughout the implant as shown by the intense immunoperoxidase staining (Figure 5). However, TN-C presence within the elastin implants was almost completely suppressed by BB-1101 in the site-specific delivery group, and was significantly reduced in the systemic delivery group (Figure 5), strongly indicating that TN-C production is dependent on MMP activity. Both control and BB-1101 groups showed comparable alkaline phosphatase activity within the implant (Figure 6), however, at this time we do not know which cell types show alkaline phosphatase activity. Nevertheless, alkaline phosphatase activity within the elastin implant was not altered by BB-1101 administration indicating that MMPs and TN-C may regulate calcification via an alternative pathway.

Figure 4. Gelatin zymography for the extracts of elastin implants (7 days). **A:** Local BB-1101 injection. **B:** Local control injection. **C** and **D** are identical to **A** and **B** except BB-1101 ($3 \mu\text{mol/L}$) was added to the substrate buffer while developing the zymograms.

Inhibition of MMP Reduces Elastin Calcification

Site-specific delivery of BB-1101 significantly inhibited calcification of elastin implants at 7 days (Table 1) with a fourfold decrease in the elastin calcium content in the BB-1101 group explants as compared to control injections ($5.43 \pm 1.03 \mu\text{g/mg Ca}$ for BB-1101 group *versus* 21.71 ± 1.19 for control group, $P < 0.001$). Alizarin Red staining clearly showed that the elastin fibers were

Figure 5. Immunohistochemical localization of TN-C within the elastin explants: MMP-inhibitor studies. Control injections show intense TN-C staining (**A**) around the calcified elastin (**arrow**); site-specific injections of BB-1101 almost completely suppressed the TN-C staining within the implants (**B**); distal BB-1101 injections also significantly reduced TN-C staining within the implants (**C**). Original magnifications, **A–C**, $\times 40$.

Figure 6. Alkaline phosphatase staining. **A:** Control injection (local). **B:** BB-1101 injection (local). Original magnification, **A** and **B**, $\times 10$. EL, elastin implant; CP, surrounding capsule.

Figure 7. Alizarin Red staining for MMP inhibitor studies: the control injection (local) showed severe calcification (**A**); site-specific BB-1101 injections showed significantly lower calcification within the implant. Original magnifications, **A** and **B**, $\times 10$.

Table 1. Seven-Day Rat Subdermal Calcification Data, MMP-Inhibitor Studies

Group (n = 10)	Ca ($\mu\text{g}/\text{mg}$)	P ($\mu\text{g}/\text{mg}$)	Ca:P molar ratio	Increase in rat weights, %	Serum calcium levels ($\mu\text{g}/\text{ml}$)
Control injection (local)	21.71 $\pm$ 1.03*	7.09 $\pm$ 0.37	2.45	66 $\pm$ 3	81.20 $\pm$ 2.33
BB-1101 injection (local)	5.43 $\pm$ 1.03*	2.52 $\pm$ 0.35	1.72	68 $\pm$ 6	89.83 $\pm$ 4.93
Control injection (distal)	28.08 $\pm$ 5.82 [†]	19.36 $\pm$ 2.39	1.16	69 $\pm$ 5	92.00 $\pm$ 4.58
BB-1101 injection (distal)	16.93 $\pm$ 2.56 [†]	10.66 $\pm$ 1.78	1.27	64 $\pm$ 2	97.53 $\pm$ 0.81

* $P < 0.001$; [†] $P < 0.05$.

heavily calcified in the control group, whereas in BB-1101 local therapy group the calcification was scarce, with few fibers showing initial calcification deposits (Figure 7). The systemic administration of BB-1101 also significantly reduced elastin calcification (28.07 $\pm$ 5.81 control versus 16.92 $\pm$ 2.56 BB-1101 group, $P < 0.05$), although less than the site-specific administration (Table 1), with obvious calcification per Alizarin Red staining (data not shown). No adverse side effects of this drug therapy were seen on the development of rats as assessed by body weights at the time of explantation (Table 1). Furthermore, serum calcium levels of the rats were not altered by BB-1101 therapy (Table 1).

Discussion

Pathological calcification is a major terminal process in a variety of cardiovascular diseases. In particular, elastin present in arteries is prone to calcification.^{2,4,35–41} In the present study, we show that MMPs, TN-C, and alkaline phosphatase are expressed within elastin implants at an early stage of calcification. Moreover, we also show that *in vivo* administration of an MMP inhibitor in rats leads to a significant reduction in elastin implant calcification with associated reduction in TN-C.

MMPs are a family of zinc-dependent enzymes involved in tissue morphogenesis and remodeling of connective tissue. In the present study, intense MMP activity within the elastin implant was seen at 7 days. This high MMP activity could lead to the degradation of elastin, thereby generating elastin peptides. Elastin peptides have been shown chemotactic for fibroblasts, smooth muscle cells, and monocytes.^{42,43} Elastin peptides contain no RGD sequences and elastin does not interact with integrins. A 67-kD elastin-binding protein, known as elastin-laminin receptor, recognizes a hydrophobic hexapeptide, VGVAPG, which repeats in the elastin molecule.⁴⁴ This 67-kD elastin-binding receptor has been shown to be present on a variety of differentiated cells including chondrocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, monocytes, and macrophages.^{45–49} Activation of this receptor (because of elastin binding) triggers several cellular reactions such as modulation of the biosynthesis of connective tissue macromolecules, increase synthesis and release of protease including MMPs, modification of ion fluxes, and cell proliferation and apoptosis.^{48–51} Thus, in our elastin implants, it is possible that initial release of elastin peptides because of MMPs can trigger activation of this receptor on the fibroblasts and monocytes and cause several down-stream events in-

cluding induction of MMPs and TN-C expression, as well as an increase in calcium ion fluxes within the cells. It has already been shown that rabbits, after injected with elastin peptides, develop aortic calcification.⁵²

When the elastin fibers were implanted subdermally, we observed a low-level inflammatory response by 7 days as assessed by scant macrophage staining. The surrounding tissue capsule and elastin implant was infiltrated with microvessels and with numerous monocytes and fibroblast-like cells (Figure 3). This represents a typical wound-healing response at this early stage of implantation. In our previous study, when the implant time was extended for 21 days, only occasional monocytes were observed within the implant.⁵ In the present study, MMP and TN-C activity observed throughout the implant at 7 days might be because of the expression of these proteins by fibroblasts or monocytes. The cell-specific expressions of MMP and TN-C are currently under investigation. However, we have shown previously that porcine aortic valve tissue when implanted subdermally calcify to the same extent in congenitally athymic, T-cell deficient (nude) mice as implants in immunologically competent hosts.⁵³ Thus, inflammatory response may play a limited role in the calcification process.

In this study, we also determined whether early inhibition of MMP activity would affect elastin calcification. Thus, we decided to block the activity of MMPs and study the down-stream events including TN-C production, alkaline phosphatase activity, and calcification. Accordingly we used a nonspecific MMP inhibitor, BB-1101. Such hydroxamate-based MMP inhibitors have been successfully used to prevent MMP activity in animal models as well as in humans.⁵⁴ Although BB-1101 inhibits activity of almost all MMPs, this was not of concern in our elastin implant studies as we see higher activities of only MMP-2 and MMP-9 in our implants as per immunohistochemistry and zymography. As BB-1101 modulates MMP activity, rather than expression level, we observed an intense activity of MMP-2 (both latent and active form) and MMP-9 in control group (vehicle treated) as well as in the BB-1101-treated group within elastin implants at 7 days as per immunohistochemistry and zymography. The experiments where BB-1101 was added to the substrate buffer while performing zymography clearly indicated that this drug blocks overall MMP activity.

Next, we decided to study TN-C within the elastin implants as TN-C up-regulation by MMPs has been demonstrated.^{18,19,25} Blocking MMP activity resulted in significant down-regulation of TN-C production within elastin implants. This is in agreement with a previous cell culture

study, where a specific MMP inhibitor, GM6001 also suppressed TN-C expression.¹⁸ Although TN-C expression is associated with physiological mineralization, the exact role of TN-C in pathological calcification is unknown. Our elastin implant studies show that TN-C production is dependent on MMP activation. Higher TN-C production was seen in highly calcified samples, while inhibiting MMPs activation via administration of BB-1101 led to diminished TN-C production and significant reduction in calcification. Future studies will directly evaluate the role of TN-C on elastin calcification. This study was performed with purified elastin samples and most of the purification methods reported for elastin cause partial degradation of elastin.

In conclusion, we have shown that MMPs, TN-C, and alkaline phosphatase are associated with the initial calcific deposits within elastin implants. Furthermore, we have shown that blocking of MMP activity by administration of BB-1101, significantly reduces elastin-oriented calcification. We believe that this is the first study where MMP inhibition suppressed pathological calcification.

References

- Grant RA: Content and distribution of aortic collagen, elastin and carbohydrate in different species. *J Atheroscler Res* 1967, 7:463-472
- Truter S, Rosenbaum-Fiedler J, Sapadin A, Lebowitz M: Calcification of elastic fibers in pseudoxanthoma elasticum. *Mt Sinai J Med* 1996, 63:210-215
- Niederhoffer N, Lartaud-Ijdouadiene I, Giummelly P, Duvivier C, Pesslin R, Atkinson J: Calcification of medial elastic fibers and aortic elasticity. *Hypertension* 1997, 29:999-1006
- Bobryshev YV, Lord RS, Warren BA: Calcified deposit formation in intimal thickenings of the human aorta. *Atherosclerosis* 1995, 118: 9-21
- Vyavahare N, Ogle M, Schoen FJ, Levy RJ: Elastin calcification and its prevention with aluminum chloride pretreatment. *Am J Pathol* 1999, 155:973-982
- Davidson JM, Zang MC, Zoia O, Giro MG: Regulation of elastin synthesis in pathological states. *Ciba Found Symp* 1995, 192:81-94
- Emonard H, Hornebeck W: Binding of 92 kDa and 72 kDa progelatinases to insoluble elastin modulates their proteolytic activation. *J Biol Chem* 1997, 378:265-271
- Reilly JM, Brophy CM, Tilson MD: Characterization of an elastase from aneurysmal aorta which degrades intact aortic elastin. *Ann Vasc Surg* 1992, 6:499-502
- Mechem RP, Broekelmann TJ, Fliszar CJ, Shapiro SD, Welgus HG, Senior RM: Elastin degradation by matrix metalloproteinases. Cleavage site specificity and mechanisms of elastolysis. *J Biol Chem* 1997, 272:18071-18076
- Tatsuguchi A, Fukuda Y, Ishizaki M, Yamanaka N: Localization of matrix metalloproteinases and tissue inhibitor of metalloproteinases-2 in normal human and rabbit stomachs. *Digestion* 1999, 60:246-254
- Zucker S, Hymowitz M, Conner C, Zarrabi HM, Hurewitz AN, Matrisian L, Boyd D, Nicolson G, Montana S: Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues. Clinical and experimental applications. *Ann NY Acad Sci* 1999, 878:212-227
- Ueno H, Yamashita K, Azumano I, Inoue M, Okada Y: Enhanced production and activation of matrix metalloproteinase-7 (matrilysin) in human endometrial carcinomas. *Int J Cancer* 1999, 84:470-477
- Simionescu D, Simionescu A, Deac R: Detection of remnant proteolytic activities in unimplanted glutaraldehyde-treated bovine pericardium and explanted cardiac bioprostheses. *J Biomed Mater Res* 1993, 27:821-829
- Simionescu A, Simionescu D, Deac R: Biochemical pathways of tissue degeneration in bioprosthetic cardiac valves. The role of matrix metalloproteinases. *ASAIO J* 1996, 42:M561-M567
- Galis ZS, Sukhova GK, Lark MW, Libby P: Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994, 94: 2493-2503
- Nikkari ST, Geary RL, Hatsukami T, Ferguson M, Forough R, Alpers CE, Clowes AW: Expression of collagen, interstitial collagenase, and tissue inhibitor of metalloproteinases-1 in restenosis after carotid endarterectomy. *Am J Pathol* 1996, 148:777-783
- Mackie EJ, Ramsey S: Expression of tenascin in joint-associated tissues during development and postnatal growth. *J Anat* 1996, 188: 157-165
- Jones PL, Crack J, Rabinovitch M: Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* 1997, 139:279-293
- Cowan KN, Jones PL, Rabinovitch M: Regression of hypertrophied rat pulmonary arteries in organ culture is associated with suppression of proteolytic activity, inhibition of tenascin-C, and smooth muscle cell apoptosis. *Circ Res* 1999, 84:1223-1233
- Zempo N, Kenagy RD, Au YP, Bendek M, Clowes MM, Reidy MA, Clowes AW: Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J Vasc Surg* 1994, 20: 209-217
- Strauss BH, Robinson R, Batchelor WB, Chisholm RJ, Ravi G, Natarajan MK, Logan RA, Mehta SR, Levy DE, Ezrin AM, Keeley FW: *In vivo* collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circ Res* 1996, 79: 541-550
- Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresch DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 1996, 85:683-693
- Sriramarao P, Steffner P, Gehlsen KR: Biochemical evidence for a homophilic interaction of the alpha 3 beta 1 integrin. *J Biol Chem* 1993, 268:22036-22041
- Prieto AL, Edelman GM, Crossin KL: Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proc Natl Acad Sci USA* 1993, 90:10154-10158
- Jones PL, Jones FS, Zhou B, Rabinovitch M: Induction of vascular smooth muscle cell tenascin-C gene expression by denatured type I collagen is dependent upon a beta3 integrin-mediated mitogen-activated protein kinase pathway and a 122-base pair promoter element. *J Cell Sci* 1999, 112:435-445
- Mackie EJ, Murphy LI: The role of tenascin-C and related glycoproteins in early chondrogenesis. *Microsc Res Tech* 1998, 43:102-110
- Moiseiwitsch JR, Raymond JR, Tamir H, Lauder JM: Regulation by serotonin of tooth-germ morphogenesis and gene expression in mouse mandibular explant cultures. *Arch Oral Biol* 1998, 43:789-800
- Tucker RP, Moiseiwitsch JR, Lauder JM: *In situ* localization of tenascin mRNA in developing mouse teeth. *Arch Oral Biol* 1993, 38:1025-1029
- Mackie EJ, Ramsey S: Modulation of osteoblast behaviour by tenascin. *J Cell Sci* 1996, 109:1597-1604
- Mackie EJ, Abraham LA, Taylor SL, Tucker RP, Murphy LI: Regulation of tenascin-C expression in bone cells by transforming growth factor-beta. *Bone* 1998, 22:301-307
- Satta J, Soini Y, Pollanen R, Paakko P, Juvonen T: Tenascin expression is associated with a chronic inflammatory process in abdominal aortic aneurysms. *J Vasc Surg* 1997, 26:670-675
- Webb CM, Zaman G, Mosley JR, Tucker RP, Lanyon LE, Mackie EJ: Expression of tenascin-C in bones responding to mechanical load. *J Bone Miner Res* 1997, 12:52-58
- Schoen FJ, Hirsch D, Bianco RW, Levy RJ: Onset and progression of calcification in porcine aortic bioprosthetic valves implanted as orthotopic mitral valve replacements in juvenile sheep. *J Thorac Cardiovasc Surg* 1994, 108:880-887
- Chen P, Toribara T, Warner H: Microdetermination of phosphorus. *Anal Chem* 1956, 28:1756-1758
- Jeziorska M, McCollum C, Woolley DE: Calcification in atherosclerotic plaque of human carotid arteries: associations with mast cells and macrophages. *J Pathol* 1998, 185:10-17
- Atkinson J: Aging of arterial extracellular matrix elastin: etiology and consequences. *Pathol Biol (Paris)* 1998, 46:555-559
- Paule WJ, Bernick S, Strates B, Nimmi ME: Calcification of implanted

- vascular tissues associated with elastin in an experimental animal model. *J Biomed Mater Res* 1992, 26:1169–1177
38. Fartasch M, Haneke E, Hornstein OP: Mineralization of collagen and elastic fibers in superficial dystrophic cutaneous calcification: an ultrastructural study. *Dermatologica* 1990, 181:187–192
39. Bestetti-Bosisio M, Cotelli F, Schiaffino E, Sorgato G, Schmid C: Lung calcification in long-term dialysed patients: a light and electronmicroscopic study. *Histopathology* 1984, 8:69–79
40. Long MM, Urry DW: On the molecular mechanism of elastic fiber calcification. *Trans Am Soc Artif Intern Organs* 1981, 27:690–696
41. Cobb CM, Howell BE, Gray RC, Weatherford TW: Potential of elastin and collagen as initiators of *in vivo* calcification. *Oral Surg Oral Med Oral Pathol* 1976, 41:24–31
42. Robert L, Jacob MP, Labat-Robert J: Cell-matrix interactions in the genesis of arteriosclerosis and atheroma. Effect of aging. *Ann NY Acad Sci* 1992, 673:331–341
43. Robert L, Jacob MP, Fulop T: Elastin in blood vessels. *Ciba Found Symp* 1995, 192:286–299
44. Mecham RP: Receptors for laminin on mammalian cells. *FASEB J* 1991, 5:2538–2546
45. Hinek A, Wrenn DS, Mecham RP, Barondes SH: The elastin receptor: a galactoside-binding protein. *Science* 1988, 239:1539–1541
46. Peterszegi G, Robert AM, Robert L: Presence of the elastin-laminin receptor on human activated lymphocytes. *C R Acad Sci III* 1996, 319:799–803
47. Peterszegi G, Mandet C, Texier S, Robert L, Bruneval P: Lymphocytes in human atherosclerotic plaque exhibit the elastin-laminin receptor: potential role in atherogenesis. *Atherosclerosis* 1997, 135:103–107
48. Jacob MP, Fulop Jr T, Foris G, Robert L: Effect of elastin peptides on ion fluxes in mononuclear cells, fibroblasts, and smooth muscle cells. *Proc Natl Acad Sci USA* 1987, 84:995–999
49. Varga Z, Jacob MP, Robert L, Fulop Jr T: Identification and signal transduction mechanism of elastin peptide receptor in human leukocytes. *FEBS Lett* 1989, 258:5–8
50. Peterszegi G, Texier S, Robert L: Cell death by overload of the elastin-laminin receptor on human activated lymphocytes: protection by lactose and melibiose. *Eur J Clin Invest* 1999, 29:166–172
51. Peterszegi G, Texier S, Robert L: Human helper and memory lymphocytes exhibit an inducible elastin-laminin receptor. *Int Arch Allergy Immunol* 1997, 114:218–223
52. Robert L, Grosogeat Y, Robert AM, Robert B: Mechanism of calcification of elastic tissue. Induction of a typical arteriosclerotic lesion by immunization of rabbits with purified elastin. *Isr J Med Sci* 1971, 7:431–432
53. Levy RJ, Schoen FJ, Howard SL: Mechanisms of calcification of porcine bioprosthetic aortic valve cusps: role of T-lymphocytes. *Am J Cardiol* 1983, 52:629–631
54. Brown PD: Synthetic inhibitors of matrix metalloproteinases. *Matrix Metalloproteinases*. Edited by WC Park, RP Mecham. San Diego, Academic Press, 1998, pp 243–261