Elastin Calcification in the Rat Subdermal Model Is Accompanied by Up-Regulation of Degradative and Osteogenic Cellular Responses

Jeoung Soo Lee, Dina M. Basalyga,
Agneta Simionescu, Jason C. Isenburg,
Dan T. Simionescu, and Narendra R. Vyavahare

From the Department of Bioengineering, Clemson University,
Clemson, South Carolina

Calcification of vascular elastin occurs in patients with arteriosclerosis, renal failure, diabetes, and vascular graft implants. We hypothesized that pathological elastin calcification is related to degenerative and osteogenic mechanisms. To test this hypothesis, the temporal expression of genes and proteins associated with elastin degradation and osteogenesis was examined in the rat subdermal calcification model by quantitative real-time reverse transcription-polymerase chain reaction and specific protein assays. Purified elastin implanted subdermally in juvenile rats exhibited progressive calcification in a time-dependent manner along with fibroblast and macrophage infiltration. Reverse transcription-polymerase chain reaction analysis showed that relative gene expression levels of matrix metalloproteinases (*MMP-2* and *MMP-9*) and *transforming growth factor- β 1* were increased in parallel with calcification. Gelatin zymography showed strong MMP activities at early time points, which were associated with high levels of soluble elastin peptides. Gene expression of *core binding factor α -1*, an osteoblast-specific transcription factor, increased in parallel with elastin calcification and attained ~9.5-fold higher expression at 21 days compared to 3 days after implantation. Similarly, mRNA levels of the bone markers *osteopontin* and *alkaline phosphatase* also increased progressively, but *osteocalcin* levels remained unchanged. We conclude that degenerative and osteogenic processes may be involved in elastin calcification. (Am J Pathol 2006; 168:490–498; DOI: 10.2353/ajpath.2006.050338)

mainly in the intima associated with lipid deposition, macrophages, and activated vascular smooth muscle cells, whereas medial calcification, also known as Mönckeberg's medial sclerosis, occurs in the media and is typically associated with elastin.¹ Vascular calcification is known to cause decreased elasticity, which is partially responsible for disorders related to elastic fibers.² In the early stages of medial calcification, morphology differs distinctly from intimal calcification, appearing as linear deposits along elastic lamina throughout most of the medial width, and in advanced lesions, in which the media is filled with circumferential rings of mineral deposits. At later stages of the disease, osteocytes are seen within bone trabeculae with apparent bone marrow formation.³ Despite its clinical significance, the molecular mechanisms regulating calcification are still unclear. Several recent studies indicate that ectopic mineralization is a highly regulated active process exhibiting many characteristics of bone formation.^{4,5} In this study, we focused on evaluating the cellular and molecular mechanisms of elastin-specific calcification in an animal model.

Elastin is a major component of the extracellular matrix in cardiovascular connective tissues. Matrix metalloproteinases (MMPs) are expressed in normal physiological processes such as wound healing and angiogenesis, but increased levels of MMPs have also been identified in many cardiovascular pathologies.⁶ MMP-9 and MMP-2 bind and degrade insoluble elastin to generate soluble peptides.⁷ These elastin peptides can interact with a 67-kd transmembrane protein, the elastin laminin receptor (ELR),⁸ which is present on the surface of most cells. Activation of ELR by elastin peptides triggers diverse biological activities in various cell types including synthesis and release of elastase, liberation of free radicals, increased Ca^{2+} influx in endothelial cells, NO-dependent vasorelaxation, proliferation of arterial smooth muscle

Supported by the National Institutes of Health (grant HL-61652).

Accepted for publication October 6, 2005.

Address reprint requests to Narendra R. Vyavahare, Ph.D., Cardiovascular Implant Research Laboratory, Department of Bioengineering, 501 Rhodes Engineering Research Center, Clemson University, Clemson, SC 29634. E-mail: narenv@clemson.edu.

Vascular calcification occurs at two distinct sites in the blood vessels. In atherosclerosis, calcification occurs

cells, chemotaxis of monocytes and fibroblasts, and apoptosis.^{9–13} Taken together, these data suggest a possible correlation between elastin degradation, activation of ELR, and elastin calcification, but the mechanisms that link these processes are as yet unknown. We have reported that MMP-mediated elastin degradation is the initial step in elastin calcification in the rat subdermal implantation model and that inhibition of MMPs leads to significant reduction in calcification.^{14,15} Moreover, we have recently shown in an abdominal aorta injury model in rats and MMP-knockout mice that MMP-mediated elastin degradation precedes elastin calcification and that the presence of active MMP-2 and MMP-9 are required for elastin calcification.¹⁶

We hypothesized that pathological elastin calcification is governed by two processes: enzyme-mediated elastin degeneration and cell-facilitated ectopic osteogenesis. As a result of elastin degradation, soluble peptides are released locally, where they activate quiescent cells, resulting in ELR and MMP up-regulation and enhanced matrix degradation. The degraded elastin-rich extracellular matrix becomes a calcification-prone substrate. We further hypothesized that elastin degradation also induces differentiation of nonbone cells into osteoblast-like cells, which secrete bone proteins that promote elastin calcification.¹⁷ To evaluate this hypothesis, we examined the expression of genes implicated in elastin degeneration such as *MMP-2*, *MMP-9*, *ELR*, and *transforming growth factor (TGF- β 1)* as well as osteogenesis-associated genes such as *core binding factor- α -1 (CBFA-1)*, *osteocalcin (OCN)*, *alkaline phosphatase (ALP)*, and *osteopontin (OPN)* using real-time reverse transcription-polymerase chain reaction (RT-PCR) in a time-course study in a rat subdermal elastin calcification model. We also evaluated soluble elastin peptide production and MMP activity, as markers of elastin degradation, as well as ALP enzyme activity and OCN secretion, as markers of osteoblastic differentiation. In the present study, we report that elastin remodeling and osteogenesis may be involved in elastin calcification.

Materials and Methods

Preparation of Pure Porcine Aortic Elastin

Porcine hearts were obtained from a local slaughterhouse and transported to the laboratory on ice. A supra-valvular aortic segment (3 to 4 cm) was dissected, cleaned to remove fat and adherent tissues, and rinsed in cold saline. Aortic elastin was purified by autoclaving, as previously described by Partridge and Keeley.¹⁸ Briefly, each aorta was cut into 2-mm strips, rinsed with distilled water, and shredded using a blender. The shredded aorta was washed with cold saline to remove soluble proteins until no protein was detected by BCA assay (Pierce, Rockford, IL). The washed aorta was autoclaved four times in distilled water (1 hour per cycle) and washed with distilled water until no protein was detected in the solution by BCA assay. This was then followed by defatting the aorta with ethanol and diethyl ether and lyophilization.

This procedure extracts all cellular materials, collagenous and noncollagenous components, including elastin-associated glycoproteins, leaving pure elastin intact.

Subdermal Implantation of Elastin

Juvenile male Sprague-Dawley rats (21 days old, 35 to 40 g; Harlan, Indianapolis, IN) were anesthetized with acepromazine (0.5 mg/kg; Ayerst Laboratories, Inc., Rouse Point, NJ) and maintained on isoflurane gas (2 to 2.5%) throughout surgery. A small incision was made on the back of the rats, and three subdermal pouches were formed by blunt dissection. Each rat received three 30- to 40-mg elastin implants (one per pouch), which were rehydrated in sterile saline 1 to 2 hours before implantation. Four rats were sacrificed by CO₂ asphyxiation at each time point (3, 7, 14, and 21 days after implantation), and the implants were retrieved along with the surrounding fibrous capsule. One explant from each rat was frozen in OCT (Sakura Finetek, Zoeterwoude, The Netherlands) on dry ice and stored at -80°C for immunohistochemistry; another explant was frozen on dry ice for protein analysis. The third explant from each rat was stored in RNeasy (Qiagen, Crawley, UK) for quantitative real-time RT-PCR. Small segments from each explant were frozen on dry ice for calcium and phosphorous analysis. As a control, uninjured subdermal tissue samples were collected from four rats and divided into two segments. One from each rat was stored in RNeasy for quantitative real-time RT-PCR and the other was frozen on dry ice for zymography. As a control medical grade polyester fabric (Dacron) samples (30 to 40 mg each, 4×4 mm pieces) were implanted subdermally in juvenile rats ($n = 4$) and explanted at 21 days as described above. Animal experiments were conducted according to National Institutes of Health guidelines for the care and use of laboratory animal (NIH publication no. 86-23, revised 1996). The animal protocol was approved by the Animal Research Committee at Clemson University.

Calcium and Phosphorous Determination

Analysis of calcium and phosphorous content in rat subdermal explants was performed by previously described procedures.¹⁹ Briefly, lyophilized elastin explants (16 to 23 mg) were placed in 1 ml of 6 N HCl and hydrolyzed in a boiling water bath for 8 hours. Samples were evaporated under a continuous stream of nitrogen gas and residual material dissolved in 1 ml of 0.01 N HCl. Calcium content in explants was determined ($n = 8$ per time point) with an atomic absorption spectrophotometer (model 3030; Perkin-Elmer, Norwalk, CT). Phosphorous content ($n = 8$ per time point) was measured on the same acid hydrolysates using the molybdate complexation assay.²⁰

Immunohistochemical (IHC) Staining for Characterization of Cell Infiltrates

Explants embedded in OCT were cryosectioned (6 μm) and mounted onto poly-L-lysine-coated glass slides. Sections were fixed in cold acetone for 5 minutes and blocked with 0.3% hydrogen peroxide in 0.3% normal sera in Tris-buffered saline for 5 minutes to neutralize endogenous peroxidase activity. Sections were incubated with mouse-derived primary antisera directed against rat monocytes/macrophages (1:200 dilution, MAB1435; Chemicon, Temecula, CA), vimentin (1:5000 dilution; Sigma, St. Louis, MO) diluted in TNB blocking buffer (Perkin Elmer Life Sciences, Boston, MA), and α -smooth muscle actin (Sigma) for 1 hour at room temperature. TNB buffer alone was used as negative control. Staining was visualized using rat-adsorbed biotinylated anti-mouse IgG secondary antibody (5 $\mu\text{g}/\text{ml}$; Vector Laboratories, Burlingame, CA), avidin-biotin-horseradish peroxidase complex (Vectastain Elite kit, Vector Laboratories), and diaminobenzidine substrate (Vector Laboratories). Sections were counterstained with hematoxylin and mounted. IHC for macrophages, fibroblasts, and pericytes was also performed on paraffin-embedded sections of uninjured rat skin as a control.

Alizarin Red Staining for Mineralization

Mineralization in elastin implants and Dacron implants was examined by Alizarin red staining.¹⁴ Frozen sections were warmed to room temperature, fixed in 95% ethanol for 30 seconds, stained for 3 minutes at room temperature with 1% Alizarin red solution, and rinsed with distilled water. Sections were counterstained with 1% light green solution for 8 to 10 seconds and rinsed with distilled water and mounted.

Gelatin Zymography for MMP Detection

Proteins from elastin explants with associated capsules and control rat subdermal tissue were extracted in a guanidine buffer, dialyzed, and analyzed in triplicates by gelatin zymography using 10 μg of protein per lane (as per BCA assay), as described before.²¹ Intensity of MMP bands (white on dark background) were evaluated by densitometry using LabImage software (Labsoft Diagnostics AG, Halle, Germany) and expressed as relative density units (RDUs) normalized to protein content.

Enzyme-Linked Immunosorbent Assay (ELISA) for Elastin Peptides

Quantitation of soluble elastin peptides was performed in the same extracts that were used for zymography by a competitive ELISA as described by Wei and colleagues²² with some modifications. Wells of microtiter plates were coated with 2 $\mu\text{g}/\text{ml}$ of elastin peptides (CB573; Elastin Products Company Inc., Owensville, MO). Simultaneously, in a separate plate (precoated with 0.5% bovine

serum albumin), samples were mixed 1:1 (v/v) with a 1:500 dilution of rabbit anti-bovine neck elastin antibody (PR403, Elastin Products Company Inc.). This primary antibody is specific for bovine and porcine elastin but does not cross-react with rat elastin.

After overnight incubation of both plates at 4°C, contents of each sample-antibody mixture well were transferred to corresponding wells in the elastin peptide-coated plate and allowed to react for 30 minutes at 37°C, followed by secondary antibody (anti-rabbit IgG peroxidase conjugate, diluted 1:2000). After 60 minutes at 37°C, peroxidase activity was detected using o-phenylene diamine hydrochloride as a substrate at 490 nm. All washes between steps and dilutions of samples and antibodies were performed in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% bovine serum albumin. Extracts obtained from unimplanted elastin served as controls. All assays were done in triplicates and concentrations of soluble elastin peptides were calculated from a standard curve obtained with 0 to 2 $\mu\text{g}/\text{ml}$ of competing elastin peptides and expressed as μg per mg protein.

Assays for ALP, OCN, and CBFA Protein Expression

Elastin explants with associated capsules were homogenized in RIPA extraction buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4) including protease inhibitor cocktail (Sigma). Homogenized explants were centrifuged at 13,000 rpm for 15 minutes, supernatants were collected, and protein concentrations were determined using a BCA assay kit (Pierce). ALP activities were examined with *p*-nitrophenyl phosphate as a substrate (procedure no. 104; Sigma Diagnostic Inc., St. Louis, MO). Enzyme activity was calculated using a *p*-nitrophenol standard curve and expressed as Sigma U per mg protein. The OCN ELISA was performed using a rat OCN EIA kit (Biomedical Technologies Inc., Stoughton, MA) and following the manufacturer's instructions. Rat OCN antibody-precoated well strips were incubated with samples at 4°C overnight and reacted with OCN antiserum at 37°C for 1 hour. Donkey anti-goat IgG peroxidase conjugate was used as secondary antibody and 3,3',5,5'-tetramethyl benzidine was used as the peroxidase substrate. Levels of OCN were calculated using a rat OCN standard curve and normalized to total protein concentration. CBFA-1 was assayed in elastin explants using the TransAM AML-3/Runx2 transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Levels of CBFA-1 were calculated using an osteoblast nuclear extract standard curve and normalized to total protein.

RNA Isolation and Characterization

Total RNA was isolated at predetermined time points from elastin-explanted and uninjured rat subdermal tissue us-

Table 1. Primers for Real-Time RT-PCR

Gene	Primer (forward)	Primer (reverse)	Product size (bp)	Accession no.
$\beta 2$ -MG	CGTGATCTTTCTGGTGCTTGTC	ACGTAGCAGTTGAGGAAGTTGG	123	NM_012512
MMP-2	AGGGCACCTCCTACACAGC	CAGTGGACATAGCGGTCTCG	126	NM_031054
MMP-9	GTGACACCGCTCACCTTCAC	GCGTGTGCCAGTAGACCATC	122	NM_031055
ELR	GTCAGCGTCATCTCCTCCAG	GAAGGTCCAGGTGTGAAGC	105	NM_017138
TGF- $\beta 1$	CGTGGCTTCTAGTGCTGACG	ACTGGCGAGCCTTAGTTTGG	137	NM_021578
CBFA-1	CAACCACAGAACCACAAGTGC	CACTGACTCGGTTGGTCTCG	120	AF053950
OCN	TATGGCACCACCGTTTAGGG	CTGTGCCGTCCATACTTTCG	123	NM_013414
ALP	TCCCAAAGGCTTCTTCTTGC	ATGGCCTCATCCATCTCCAC	108	J03572
OPN	CTGCCAGCACACAAGCAGAC	TCTGTGGCATCGGGATACTG	145	AB001382

ing RNeasy fibrous tissue kit (Qiagen, Valencia, CA). RNA quality and quantity were evaluated for each sample using the RNA 6000 Nano Assay, Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Germantown, MD). The purity of RNA was determined by RIN (RNA integrity number) software algorithm (Agilent Technologies, Inc.), which allows for the classification of eukaryotic total RNA based on a numbering system from 1 to 10, with 1 being the most degraded RNA and 10 being the most intact RNA. The RINs of all RNAs extracted were greater than 9 (data not shown).

Real-Time RT-PCR Analysis

Reverse transcription reactions were performed with 1 μ g of total RNA for each 20- μ l RT reaction using Moloney murine leukemia virus reverse transcriptase with oligo (dT) primers (RetroScript Kit; Ambion). Target-specific PCR primers were designed using Primer3 software and synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Primer sets, gene accession numbers, and PCR product sizes are listed in Table 1. Real-time PCR amplifications were performed using SYBR Green PCR kit (Qiagen) in a Rotorgene 3000 thermal cycler (Corbett Research, Mortlake, NSW, Australia), and the cycle number at which the amplification plot crosses the threshold was calculated (C_T). The $2^{-\Delta\Delta C_T}$ method was used to analyze the relative changes in gene expression from real-time quantitative PCR using $\beta 2$ -microglobulin ($\beta 2$ -MG) as a housekeeping gene. Minus RT reactions performed on a representative subset of samples demonstrated that genomic DNA contamination was insignificant (data not shown). Reaction specificities were routinely verified by product purification, agarose gel electrophoresis, as well as routine melting curve analysis. The identities of PCR products were determined by automated sequencing at Arizona State University DNA Analysis Facility. The levels of gene expression were presented as the change in expression of a given time point (X) relative to 3 days as time 0 in a time-course study using the $2^{-\Delta\Delta C_T}$ method²³ as follows: $\Delta\Delta C_T = (C_T \text{ target gene} - C_T \beta 2\text{-MG})_{\text{Time X}} - (C_T \text{ target gene} - C_T \beta 2\text{-MG})_{\text{Time 0}}$.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Statistical significance of value at a given time point as compared to the

3-day time point was determined by Student's *t*-test with statistical significance defined as $P < 0.05$.

Results

Assessment of Calcification

Calcium content in elastin explants increased progressively with time ($P < 0.05$ for all time points) and reached $133.65 \pm 5.82 \mu\text{g Ca/mg dry explant}$ at 21 days. Phosphorous content also increased in parallel with calcium content (Figure 1). The molar ratios of Ca/P were 2.5, 1.6, 1.5, and 1.4 at 3, 7, 14, and 21 days, respectively, suggesting the presence of poorly crystalline hydroxyapatite deposition. We have previously analyzed the mineral associated with elastin in this animal model by X-ray diffraction and have shown that it resembles hydroxyapatite.²⁴ Control Dacron implants did not show any calcification in this model ($0.8 \pm 0.09 \mu\text{g Ca/mg dry explants}$ at 21 days).

Characterization of Cellular Infiltration and Mineralization

To evaluate the incidence of infiltrating cells in the implants, IHC was performed to identify macrophages, fibroblasts, and pericytes/activated myofibroblasts. Vimentin-positive fibroblasts were observed throughout the capsule and within the elastin implants and they were apparently the dominant cells alongside with infiltrating

Figure 1. Time course of elastin calcification in the rat subdermal model. The calcium and phosphorous contents are expressed as $\mu\text{g/mg dry explant}$ and are presented as means $\pm$ SEM ($n = 8$).

Figure 2. Cellular infiltration of elastin implants. At 21 days after implantation, IHC staining showed that the elastin implant (EL) was invaded by numerous macrophage/monocytes (A), fibroblast-like cells (B), and pericytes/activated fibroblasts outlined by arrows (C). IHC staining for macrophages/monocytes (D), fibroblasts (E), pericytes/activated fibroblasts (F) is also shown for normal rat skin. Alizarin red staining showed mineralization of elastin fibers (G) at 21 days after implantation compared to no calcification in Dacron implant (DC) as a control (H). Insets show corresponding negative controls for IHC staining. Original magnifications, $\times 200$.

macrophages (Figure 2, A and B). These cells appeared to make intimate contact with the elastin fibers. α -Smooth muscle cell actin staining showed the presence of pericytes in small blood vessels forming within the capsule (Figure 2C). Normal skin samples of rats showed strong staining for fibroblasts throughout and for pericytes around the vasculature but weak staining for few scattered macrophages (Figure 2, D–F). Alizarin red staining showed that elastin fibers started to calcify at the edge of elastin implants as early as 3 days after implantation (data not shown), and significant calcium deposition was noted throughout the entire elastin implant at 21 days after implantation (Figure 2G) whereas no calcification was observed in Dacron implant at 21 days after implantation (Figure 2H).

Characterization of Elastin Degradation

Relative gene expression levels for *MMP-2*, *MMP-9*, and *TGF- β 1* were up-regulated in elastin subdermal implants with increasing elastin calcification (Figure 3). mRNA levels of *TGF- β 1* and *MMP-9* significantly increased at 7 days and remained at high levels at 2 and 3 weeks, respectively ($P < 0.05$), in parallel with increasing elastin calcification. In the case of *MMP-2* and *ELR*, mRNA levels were significantly increased ($P < 0.05$) at 7 and 14 days

as compared to 3 days after implantation and then decreased at 21 days; overall the levels were lower than control rat subdermal tissue. To evaluate MMP activities, gelatin zymography was performed on protein extracts

Figure 3. Time course of gene expression associated with elastin degradation and remodeling in elastin implants. Isolated RNA was analyzed by real-time RT-PCR for *MMP-2*, *MMP-9*, *ELR*, *TGF- β 1*, and β 2-microglobulin. Gene expression levels are presented as the change in expression of a given time point relative to 3 days in a time course study using the $2^{-\Delta\Delta C_T}$ method and normalized to β 2-MG. Data points represent means $\pm$ SEM ($n = 4$). * $P < 0.05$.

Figure 4. Protein expression associated with elastin degradation. **A:** MMP activities from elastin explants were assayed by gelatin zymography (top) and expressed as RDU (relative density units)/mg protein (bottom). Data points represent means $\pm$ SEM ($n = 3$). **B:** Content of soluble elastin peptides was determined by ELISA and expressed as μ g elastin peptides/mg protein. Data points represent means $\pm$ SEM ($n = 3$).

obtained at various time points from elastin explants and control rat subdermal tissue (Figure 4A). At 3 days, MMP-9 activity was the strongest and then decreased slightly with time. MMP-2 activity was also increased at earlier time points and then decreased with time. Overall MMP levels were significantly higher in elastin implants as compared to control subdermal tissue. The content of soluble elastin peptides as a consequence of elastin degradation in elastin explants was determined by ELISA assay and showed the highest levels at 7 days, in parallel with the MMP activities in elastin explants (Figure 4B).

Characterization of Osteogenic Signals

Real-time RT-PCR was performed on the isolated RNA from elastin explants at different time points to evaluate osteogenic gene expression in cells associated with elastin implants. Relative gene expression of *CBFA-1*, *OPN*, and *ALP* increased in elastin subdermal implants with increasing elastin calcification, but *OCN* levels, a late marker of calcification in osteogenesis, remained unchanged (Figure 5). In particular, mRNA levels of *CBFA-1*, an osteoblast-specific transcription factor, increased significantly at all time points compared to 3 days and attained ~ 9.5 -fold higher expression at 21 days

Figure 5. Time course of osteoblast-specific gene expression in elastin implants. Isolated RNA was analyzed by RT-PCR for *CBFA-1*, *OCN*, *OPN*, and *ALP*, and $\beta 2$ -microglobulin. The levels of mRNA expression are presented as the change in expression relative to 3 days in a time course study using the $2^{-\Delta\Delta CT}$ method and normalized to $\beta 2$ -microglobulin. Data points represent means $\pm$ SEM ($n = 4$). * $P < 0.05$.

than that at 3 days ($P < 0.05$). ELISA quantitation detected *CBFA-1* protein levels in elastin explants at 14 and 21 days after implantation (12.62 ± 0.66 and 6.88 ± 3.27 μ g nuclear extract equivalent of osteoblasts/mg protein, respectively).

mRNA levels of *OPN* and *ALP* were higher at all time points relative to those at 3 days. ALP activity and *OCN* protein production in elastin explants as markers of osteogenic remodeling were also evaluated. ALP activity increased ~ 1.32 -, 5.44 -, and 4.07 -fold at 7, 14, and 21 days, respectively, compared to 3 days (Figure 6A). *OCN* levels were, however, not significantly changed ($P > 0.05$) in parallel with mineralization (Figure 6B). Overall, protein secretion of ALP and *OCN* matched with mRNA levels at various time points.

Discussion

Pathological vascular elastin-specific calcification is seen in a variety of diseases including patients with arteriosclerosis, renal failure and diabetes, implanted vascular grafts, and aging aortic stenosis.²⁵⁻²⁷ We have previously reported that purified porcine aortic elastin, when

Figure 6. Protein expression associated with osteoblast-like cell differentiation in elastin implants. **A:** ALP activity in protein extracts of elastin explants were assayed and expressed as Sigma units/mg protein. Data points represent means $\pm$ SEM ($n = 3$). **B:** *OCN* was determined by ELISA assay and expressed as ng *OCN*/mg protein. Data points represent means $\pm$ SEM ($n = 3$). * $P < 0.05$.

implanted subdermally in rats, exhibited progressive calcification.¹⁵ The present work is an extension of previous studies to evaluate the cellular and molecular mechanisms involved in elastin calcification. We hypothesized that pathological calcification of medial arterial calcification is associated with two processes: elastin degeneration and ectopic osteogenesis. To evaluate this hypothesis, we subdermally implanted purified porcine aortic elastin in rats and evaluated gene expression accompanying elastin calcification.

MMPs have been identified in pathological conditions in many cardiovascular diseases.⁶ Longo and colleagues²⁸ reported that macrophage-derived MMP-9 and MMP-2 derived from mesenchymal cells (smooth muscle cells and fibroblasts) work in concert to produce abdominal aortic aneurysms, which involves elastic lamina degeneration. MMP-9 and MMP-2 degrade insoluble elastin to soluble peptides. Elastin peptides have been shown to activate the ELR.⁸ The ELR was identified on lymphocytes, macrophages, granulocytes, smooth muscle cells, and endothelial cells.^{29,30} Activation of this receptor by binding of elastin peptides triggers diverse biological activities. These peptides stimulate fibroblast adhesion to elastin fibers,³¹ regulate cellular proliferation,³² and are chemotactic for several cell types, such as monocytes³³ and fibroblasts.⁹ Substantial infiltration of elastin implants by macrophages/monocytes and fibroblasts, observed by IHC staining (Figure 2, A and B), resulted in higher cell densities than those observed in normal rat skin (Figure 2, D and E). Increased mRNA expression of *MMP-9* and *MMP-2* was observed at all time points compared to 3 days as an earliest time point of calcification (Figure 3). Both *MMP-9* and *MMP-2* showed high enzyme activities at early time points in calcification (Figure 4A), and the level of degraded elastin peptides in elastin explants was also increased in parallel with MMP expression (Figure 4B). The mRNA level of *ELR* was also increased significantly at 7 and 14 days compared to 3 days although the expression levels in implants were lower than those found in normal skin. Thus, it is possible that MMP-mediated degradation of elastin after implantation leads to binding of soluble elastin peptides to ELR on both fibroblasts and macrophages in our implants. The mRNA level of *TGF- β 1* was also increased in parallel with *MMP-2* and *MMP-9* gene expression. Several studies have demonstrated multiple interactions between MMPs and *TGF- β 1* that suggest simultaneous expression of these molecules may promote elastin calcification through a positive-feedback mechanism. *MMP-2* and *MMP-9* can activate *TGF- β 1* by cleaving its latent form,³⁴ and *TGF- β 1* has been shown to induce an elevated level of *MMP* gene expression.^{35,36} *TGF- β 1* promotes osteoprogenitor cell proliferation and osteogenesis. *TGF- β 1* also stimulates ALP activity in cells.³⁷ Thus, increased expression of *TGF- β 1* in our implants may promote osteogenesis.

The occurrence of ectopic osteogenesis process was confirmed by the up-regulated expression of bone-specific genes such as *CBFA-1*, *OCN*, *OPN*, and *ALP* in elastin implants. Of particular interest, progressive and sustained increases in the expression of the osteoblast-

specific transcription factor *CBFA-1* were observed, attaining ~9.5-fold higher expression at 21 days compared to 3 days. Moreover, *CBFA-1* gene expression was observed 2.3-, 5.7-, 8.7-, and 20.2-fold higher expression at 3, 7, 14, and 21 days, respectively, than that of rat subdermal tissue. Detectable levels of *CBFA-1* protein were found at 14 days and 21 days. *CBFA-1* is the earliest and most specific marker of developmental osteogenesis. *CBFA-1* acts as an activator of transcription and can induce osteoblast-specific gene expression in fibroblasts and myoblasts.^{38,39} Significantly increased *OPN* gene expression was also observed at 7 days and 21 days compared to 3 days and control rat subdermal tissue. *OPN* is a phosphorylated protein of wide tissue distribution that is found in calcified vascular tissues.⁴⁰ Giachelli and colleagues⁴¹ reported that *OPN* expression is increased under injury and disease in many tissues and it is closely related to calcified deposits found in numerous pathologies. We also demonstrated that production of bone marker proteins such as ALP was increased in parallel with calcification on days 14 and 21. *OCN* content did not significantly change when normalized to total protein.

In the present studies, IHC analysis showed that infiltration by pericytes/activated myofibroblasts was increased in a time-dependent manner along with fibroblast and macrophage invasion. Moreover, RT-PCR and protein analysis clearly indicated the presence of osteoblast-like cells in the vicinity of calcifying elastin. Although the origin of osteoblast-like cells in calcified elastin is not clearly defined in our study, we speculate that activated fibroblasts may undergo cell differentiation and express an osteoblast-like phenotype. In support of this speculation, we have recently observed increased gene and protein expression of *CBFA-1* in primary rat skin fibroblast cell cultures exposed to elastin peptides and *TGF- β 1* for several days (data not shown). However, more research is needed to identify what specific types of cells are involved in osteogenesis in the rat subdermal implantation model.

The present work involved study of elastin calcification in the rat subdermal implantation model, which may provide some insights for elastin-specific medial calcification seen in vasculature. Elastin in native arteries is surrounded by acidic glycoproteins, including fibrillin, fibulin, and latent *TGF- β 1*-binding protein among others.⁴² These glycoproteins may protect elastin from calcification. For example, fibrillin mutant mice develop severe elastin-specific vascular calcification.⁴³ Glycoprotein levels in the arteries decrease with age, in parallel with the propensity for vascular calcification.⁴⁴ Physical (plaque formation) or biochemical (inflammatory reactions) injury may expose elastin to cells. Cells may then produce MMPs and other serine elastases to degrade elastin. Elastin peptides would then activate ELR on a variety of cells, which in turn up-regulates MMP expression by positive feedback mechanisms. MMPs are also known to degrade glycoproteins surrounding elastin, including fibrillin,⁴⁵ thus exposing more elastin for degradation. Several stimuli induce up-regulation of *MMP* gene expression and increase the degradation of matrix. The

elastin degradation may release matrix-bound cytokines such as TGF- β 1, which are chemotactic for several cells and induce inflammatory reactions.^{46,47} These released cytokines may also induce the differentiation of smooth muscle cells or fibroblasts into osteoblast-like cells, which would produce bone-specific proteins and aid the calcification process.³⁵ Vascular smooth muscle cells and pericytes, considered resting mesenchymal stem cells, can assume osteoblastic/chondrocytic phenotypes,^{3,48,49} implying a phenotypic modulation of resident vascular cells in a permissive matrix environment. TGF- β 1 has also been demonstrated to have osteogenic activity^{50,51} and to promote the calcification of aortic smooth muscle cells in culture.⁵²

There is also a possibility of cross-talk between the ELR and TGF- β /BMP-Smad intracellular pathways. The signal transduction pathway of ELR involves activation of phospholipase C by a pertussis toxin-sensitive G-protein. Phospholipase C induces the production of inositol triphosphate (IP3) leading to the increase in the intracellular free calcium.⁵³ Free calcium would then bind to calmodulin. Calmodulin has been shown to bind Smads 1 to 4 in a calcium-dependent manner.⁵⁴ The Smads were shown to play an important role in transducing specific TGF- β 1 signaling pathways. Recently, Smads were also shown to have links to CBFA-1.⁵⁵ Thus, interrelations between ELR activation and osteogenic differentiation may exist. However, more research is needed to confirm these interactions.

In conclusion, initiation and progression of elastin calcification in the rat subdermal model involves MMP-mediated elastin degeneration and localized ectopic osteogenesis. The present study may shed some light on the mechanisms of elastin calcification in medial pathological vascular calcification. Ongoing gene silencing studies will extend our investigations of these mechanisms by using delivery of MMP siRNA and CBFA-1 siRNA to block elastin degradation and osteogenesis processes in the rat subdermal model and circulatory aortic injury models.

Acknowledgment

We thank Dr. Ken Webb for his assistance with real-time RT-PCR.

References

1. Proudfoot D, Shanahan CM: Biology of calcification in vascular cells: intima versus media. *Herz* 2001, 26:245–251
2. Niederhoffer N, Lartaud-Idjouadiene I, Giummelly P, Duviols C, Pesslin R, Atkinson J: Calcification of medial elastic fibers and aortic elasticity. *Hypertension* 1997, 29:999–1006
3. Shanahan CM, Cary NR, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME: Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. *Circulation* 1999, 100:2168–2176
4. Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, Springett M, Orszulak T, Fullerton DA, Tajik AJ, Bonow RO, Spelsberg T: Human aortic valve calcification is associated with an osteoblast phenotype. *Circulation* 2003, 107:2181–2184
5. Bostrom K: Insights into the mechanism of vascular calcification. *Am J Cardiol* 2001, 88:20E–22E
6. Nagase H, Woessner JF Jr: Matrix metalloproteinases. *J Biol Chem* 1999, 274:21491–21494
7. Mecham 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
8. Hinek A, Wrenn DS, Mecham RP, Barondes SH: The elastin receptor: a galactoside-binding protein. *Science* 1988, 239:1539–1541
9. Mecham RP, Griffin GL, Madaras JG, Senior RM: Appearance of chemotactic responsiveness to elastin peptides by developing fetal bovine ligament fibroblasts parallels the onset of elastin production. *J Cell Biol* 1984, 98:1813–1816
10. Faury G, Garnier S, Weiss AS, Wallach J, Fulop Jr T, Jacob MP, Mecham RP, Robert L, Verdeti J: Action of tropoelastin and synthetic elastin sequences on vascular tone and on free Ca²⁺ level in human vascular endothelial cells. *Circ Res* 1998, 82:328–336
11. Hinek A: Biological roles of the non-integrin elastin/laminin receptor. *Biol Chem* 1996, 377:471–480
12. Mochizuki S, Brassart B, Hinek A: Signaling pathways transduced through the elastin receptor facilitate proliferation of arterial smooth muscle cells. *J Biol Chem* 2002, 277:44854–44863
13. Hance KA, Tataria M, Ziporin SJ, Lee JK, Thompson RW: Monocyte chemotactic activity in human abdominal aortic aneurysms: role of elastin degradation peptides and the 67-kD cell surface elastin receptor. *J Vasc Surg* 2002, 35:254–261
14. Vyavahare N, Jones PL, Tallapragada S, Levy RJ: Inhibition of matrix metalloproteinase activity attenuates tenascin-C production and calcification of implanted purified elastin in rats. *Am J Pathol* 2000, 157:885–893
15. Bailey MT, Pillarisetti S, Xiao H, Vyavahare NR: Role of elastin in pathologic calcification of xenograft heart valves. *J Biomed Mater Res A* 2003, 66:93–102
16. Basalyga DM, Simionescu DT, Xiong W, Baxter BT, Starcher BC, Vyavahare NR: Elastin degradation and calcification in an abdominal aorta injury model: role of matrix metalloproteinases. *Circulation* 2004, 110:3480–3487
17. Simionescu A, Philips K, Vyavahare N: Elastin-derived peptides and TGF-beta1 induce osteogenic responses in smooth muscle cells. *Biochem Biophys Res Commun* 2005, 334:524–532
18. Partridge SM, Keeley FW: Age related and atherosclerotic changes in aortic elastin. *Adv Exp Med Biol* 1974, 43:173–191
19. Schoen FJ, Levy RJ: Pathology of substitute heart valves: new concepts and developments. *J Card Surg* 1994, 9:222–227
20. Chen P, Toribara T, Warner H: Microdetermination of phosphorous. *Anal Chem* 1956, 28:1756–1758
21. Bailey M, Pillarisetti S, Jones P, Xiao H, Simionescu D, Vyavahare N: Involvement of matrix metalloproteinases and tenascin-C in elastin calcification. *Cardiovasc Pathol* 2004, 13:146–155
22. Wei SM, Erdei J, Fulop Jr T, Robert L, Jacob MP: Elastin peptide concentration in human serum: variation with antibodies and elastin peptides used for the enzyme-linked immunosorbent assay. *J Immunol Methods* 1993, 164:175–187
23. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(Delta Delta C(T))} method. *Methods* 2001, 25:402–408
24. Vyavahare N, Ogle M, Schoen FJ, Levy RJ: Elastin calcification and its prevention with aluminum chloride pretreatment. *Am J Pathol* 1999, 155:973–982
25. 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
26. Bobryshev YV, Lord RS, Warren BA: Calcified deposit formation in intimal thickenings of the human aorta. *Atherosclerosis* 1995, 118:9–21
27. David TE, Bos J: Aortic valve replacement with stentless porcine aortic valve: a pioneer series. *Semin Thorac Cardiovasc Surg* 1999, 11:9–11
28. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT: Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* 2002, 110:625–632
29. Fulop Jr T, Jacob MP, Khalil A, Wallach J, Robert L: Biological effects of elastin peptides. *Pathol Biol (Paris)* 1998, 46:497–506

30. 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
31. Groult V, Hornebeck W, Ferrari P, Tixier JM, Robert L, Jacob MP: Mechanisms of interaction between human skin fibroblasts and elastin: differences between elastin fibres and derived peptides. *Cell Biochem Funct* 1991, 9:171–182
32. Ghuyssen-Itard AF, Robert L, Jacob MP: Effect of elastin peptides on cell proliferation. *C R Acad Sci III* 1992, 315:473–478
33. Senior RM, Griffin GL, Mecham RP: Chemotactic activity of elastin-derived peptides. *J Clin Invest* 1980, 66:859–862
34. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000, 14:163–176
35. Jeziorska M: Transforming growth factor-betas and CD105 expression in calcification and bone formation in human atherosclerotic lesions. *Z Kardiol* 2001, 90(Suppl 3):23–26
36. Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001, 17:463–516
37. Yeung HY, Lee KM, Fung KP, Leung KS: Sustained expression of transforming growth factor-beta1 by distraction during distraction osteogenesis. *Life Sci* 2002, 71:67–79
38. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G: *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 1997, 89:747–754
39. Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M: *Cbfa1* isoforms exert functional differences in osteoblast differentiation. *J Biol Chem* 1999, 274:6972–6978
40. Giachelli CM: Ectopic calcification: gathering hard facts about soft tissue mineralization. *Am J Pathol* 1999, 154:671–675
41. Giachelli CM, Liaw L, Murry CE, Schwartz SM, Almeida M: Osteopontin expression in cardiovascular diseases. *Ann NY Acad Sci* 1995, 760:109–126
42. Rosenbloom J, Abrams WR, Mecham R: Extracellular matrix 4: the elastic fiber. *FASEB J* 1993, 7:1208–1218
43. Pereira L, Lee SY, Gayraud B, Andrikopoulos K, Shapiro SD, Bunton T, Biery NJ, Dietz HC, Sakai LY, Ramirez F: Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1. *Proc Natl Acad Sci USA* 1999, 96:3819–3823
44. Godfrey M, Nejezchleb PA, Schaefer GB, Minion DJ, Wang Y, Baxter BT: Elastin and fibrillin mRNA and protein levels in the ontogeny of normal human aorta. *Connect Tissue Res* 1993, 29:61–69
45. Ashworth JL, Murphy G, Rock MJ, Sherratt MJ, Shapiro SD, Shuttleworth CA, Kielty CM: Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling. *Biochem J* 1999, 340:171–181
46. Gregory AK, Yin NX, Capella J, Xia S, Newman KM, Tilson MD: Features of autoimmunity in the abdominal aortic aneurysm. *Arch Surg* 1996, 131:85–88
47. Shapiro SD: Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998, 10:602–608
48. Diefenderfer DL, Brighton CT: Microvascular pericytes express aggrecan message which is regulated by BMP-2. *Biochem Biophys Res Commun* 2000, 269:172–178
49. Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE: Vascular pericytes express osteogenic potential in vitro and in vivo. *J Bone Miner Res* 1998, 13:828–838
50. Miyazono K, Kusanagi K, Inoue H: Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol* 2001, 187:265–276
51. Bonewald LF, Dallas SL: Role of active and latent transforming growth factor beta in bone formation. *J Cell Biochem* 1994, 55:350–357
52. Jian B, Narula N, Li QY, Mohler III ER, Levy RJ: Progression of aortic valve stenosis: TGF-beta1 is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis. *Ann Thorac Surg* 2003, 75:456–465
53. Fulop Jr T, Douziech N, Jacob MP, Hauck M, Wallach J, Robert L: Age-related alterations in the signal transduction pathways of the elastin-laminin receptor. *Pathol Biol (Paris)* 2001, 49:339–348
54. von Bubnoff A, Cho KKY: Intracellular BMP signalling regulation in vertebrates: pathway or network? *Dev Biol* 2001, 239:1–14
55. Ito Y, Zhang YW: A RUNX2/PEBP2alphaA/CBFA1 mutation in cleidocranial dysplasia revealing the link between the gene and Smad. *J Bone Miner Metab* 2001, 19:188–194