Osteogenic Responses in Fibroblasts Activated by Elastin Degradation Products and Transforming Growth Factor- β 1

Role of Myfibroblasts in Vascular Calcification

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Our objective was to establish the role of fibroblasts in medial vascular calcification, a pathological process known to be associated with elastin degradation and remodeling. Rat dermal fibroblasts were treated *in vitro* with elastin degradation products and transforming growth factor (TGF)- β 1, factors usually present in deteriorated matrix environments. Cellular changes were monitored at the gene and protein level by reverse transcriptase-polymerase chain reaction, enzyme-linked immunosorbent assay, immunofluorescence, and von Kossa staining for calcium deposits. By 21 days, multicellular calcified nodules were formed in the presence of elastin degradation products and TGF- β 1 separately and to a significantly greater extent when used together. Before mineralization, cells expressed α -smooth muscle actin and large amounts of collagen type I and matrix metalloproteinase-2, characteristic features of myofibroblasts, key elements in tissue remodeling and repair. Stimulated cells expressed increased levels of core-binding factor α 1, osteocalcin, alkaline phosphatase, and osteoprotegerin, representative bone-regulating proteins. For most proteins analyzed, TGF- β 1 synergistically amplified responses of fibroblasts to elastin degradation products. In conclusion, elastin degradation products and TGF- β 1 promote myofibroblastic and osteogenic differentiation in fibroblasts. These results support the idea that elastin-related calcification involves dynamic remodeling events and suggest the possibility of a defective tissue repair process. (*Am J Pathol* 2007, 171:116–123; DOI: 10.2353/ajpath.2007.060930)

Calcification of the arterial media frequently occurs associated with chronic kidney disease, diabetes, and aging.^{1–6} Intense calcium deposition on the elastic lamellae is accompanied by proteolysis and elastic fiber degradation.^{7,8} Typical bone proteins are also expressed with this process, such as core-binding factor α 1 (Cbfa-1), osteocalcin, osteopontin, bone morphogenetic protein-2, matrix Gla-protein, and alkaline phosphatase.^{6,9,10} Osteoprotegerin (OPG), a protein apparently involved in counteracting vascular calcification and skeletal demineralization with age and osteoporosis, was also noticed in areas surrounding calcified regions in the arterial media.¹¹ It is still uncertain which vascular cells contribute to bone-protein synthesis and elastin calcification, but they are known to be positive for α -smooth muscle actin (α -SMA).¹² Vascular smooth muscle cells (SMCs) formed mineralized nodules when cultured *in vitro* for extended periods of time¹² or when specifically exposed to calcifying agents *in vitro*.¹³ We showed previously that rat aortic SMCs gain an osteogenic phenotype when treated *in vitro* with elastin degradation peptides (EDPs) and transforming growth factor (TGF)- β 1, a mediator usually present in the degraded matrix environment, even in the absence of any added calcifying agents.¹⁴ However, it is widely accepted that SMCs are primarily associated with intimal arterial calcification, related to atherosclerosis, and less so with medial calcification.^{13,15}

In injured arteries, adventitial myofibroblasts migrate toward the media and contribute to vascular remodeling and elastin calcification.¹⁶ It was shown that surgical resection of the adventitia prevents segmental medial artery calcification in a rat model.³ Myofibroblasts are

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Table 1. PCR Primers

Name	Accession no.	Forward primer	Reverse primer
Glyceraldehyde-3-phosphate dehydrogenase	AB017801	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'
β_2 -Microglobulin	NM_012512	5'-CGTGATCTTTCTGGTGCTTGTC-3'	5'-ACGTAGCAGTTGAGGAAGTTGG-3'
α -SMA	NM_031004	5'-ACTGGGACGACATGGAAAAG-3'	5'-CATACATGGCAGGGACATTG-3'
Collagen I A2	J04464	5'-GGACACAATGGATTGCAAGG-3'	5'-TAACCACTGCTCCACTCTGG-3'
MMP-2	NM_031054	5'-AGGGCACCTCCTACAACAGC-3'	5'-CAGTGGACATAGCGGTCTCG-3'
CBFA1 type II	AF053950	5'-CAACCACAGAACCACAAGTGC-3'	5'-CACTGACTCGGTTGGTCTCG-3'
Alkaline phosphatase	J03572	5'-TCCCAAAGGCTTCTTCTTGC-3'	5'-ATGGCCTCATCCATCTCCAC-3'
Osteocalcin	NM_013414	5'-TATGGCACCACCGTTTAGGG-3'	5'-CTGTGCCGTCCATACTTTTCG-3'
OPG	U94330	5'-TCCTGGCACCTAAACAGCA-3'	5'-CTACACTCTCGGCATTCACTTTGG-3'

α -SMA-positive cells that differentiate from fibroblasts in association with connective tissue injury and play a key role in matrix remodeling and tissue repair. They are capable of differentiating into a variety of cell types, including calcified vascular cells and finally osteoblasts, and are involved in the ossification of heart valves and arteries.^{3,6,17,18} Our goal was to determine whether fibroblasts, in the presence of degraded elastin and TGF- β 1, modulate into myofibroblasts and eventually into osteoblast-like cells and consequently represent a potential source of pro-mineralizing cells associated with elastin degradation.

Materials and Methods

Cell Culture and Treatments

Rat primary dermal fibroblasts were isolated using the explant technique. Cells from passage 5 were used in all experiments. Cells were cultured in six-well plates (6×10^5 /well) in Dulbecco's modified Eagle's medium (Cellgro-Mediatech, Herndon, VA) containing 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), with 100 units/ml penicillin and 100 units/ml streptomycin (Gibco, Rockville, MD), in a humidified incubator at 37°C. Cells ($n = 6$ wells/group) were treated with soluble α -elastin, a 10- to 60-kd elastin peptide mixture prepared by chemical degradation of insoluble elastin (Elastin Products Company, Owensville, MO), and recombinant human TGF- β 1 (PeproTech, Inc., Rocky Hill, NJ), as follows: 100 μ g/ml α -elastin (elastin group); 10 ng/ml TGF- β 1 (TGF group); 100 μ g/ml α -elastin and 10 ng/ml TGF- β 1 (elastin + TGF group); and medium alone (control group). Culture media were replaced every 3 days with fresh Dulbecco's modified Eagle's medium supplemented with the appropriate agents in concentrations described above. Gene and protein expression were analyzed after 10 days, as described below. Calcium deposition was evaluated by von Kossa staining of cells maintained in culture for up to 21 days.

Gene Expression

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Quality and quantity of RNA

were evaluated on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Lab-on-a-Chip kit (Agilent Technologies, Inc., Foster City, CA). One microgram of total RNA was then reverse transcribed using RetroScript kit (Ambion, Austin, TX). The cDNA sample was further amplified on a Rotorgene 3000 thermal cycler (Corbett Research, Mortlake, NSW, Australia) and using QuantiTect SYBR Green PCR kit (Qiagen), which allows for real-time quantity detection of polymerase chain reaction (PCR) products. For gene expression, we used the primer sets described in Table 1 coding for: β_2 -microglobulin,⁸ glyceraldehyde-3-phosphate dehydrogenase,¹⁹ OPG,¹⁹ collagen 1A2,²⁰ α -smooth muscle actin,²¹ Cbfa-1 type II isoform,⁸ alkaline phosphatase,⁸ osteocalcin,⁸ and matrix metalloproteinase (MMP)-2.⁸ Gene expression in each sample was normalized to the expression of a housekeeping gene and compared with control samples (cells in medium alone), using the $2^{-\Delta\Delta CT}$ method.²²

Immunofluorescence

Rat skin fibroblasts were cultured in two-well chamber slides (NuncLab-Tek II Chamber Slide System; Fisher Scientific, Pittsburgh, PA) and incubated for 10 days with α -elastin and TGF- β 1, at the doses indicated above (4×10^5 /well, $n = 4$ per group). The cultures were then fixed in 4% paraformaldehyde at room temperature for 15 minutes, permeabilized with 0.1% Triton X-100 for 2 minutes, and blocked with 1% bovine serum albumin for 1 hour. The primary antibodies used were mouse monoclonal anti- α -SMA at 1:400 dilution (Sigma, St. Louis, MO), rabbit polyclonal anti-SM22 at 1:400 dilution (GeneTex, Inc., San Antonio, TX), mouse anti-collagen type I (Sigma), and rabbit polyclonal anti-OPG at 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After overnight incubation at 4°C, cells were stained for 2 hours with AlexaFluor 488 chicken anti-rabbit IgG secondary antibody for α -SMA and collagen detection and AlexaFluor 594 anti-mouse IgG (Molecular Probes, Eugene, OR) for OPG, both diluted to 8 μ g/ml. Slides were mounted in SlowFade Antifade with 4',6-diamidino-2-phenylindole dihydrochloride blue fluorescent nuclear stain (Molecular Probes) and examined by fluorescence microscopy.

Western Blotting

Whole-cell extracts were prepared ($n = 3$ per group) by scraping and extracting the cells cultured in six-well plates in 20 mmol/L Tris, 0.5% Triton X-100, 1% sodium dodecyl sulfate, pH 7.4, and 10 μ l/ml protease inhibitor cocktail (Sigma) for 5 minutes on ice, followed by 30-second sonication and centrifugation for 15 minutes at 12,000 rpm, at 4°C. Supernatants were normalized to protein content by bicinchoninic acid assay (BCA Protein assay kit; Pierce, Rockford, IL), and 15 μ g of protein from each sample, prepared under reducing conditions, was loaded in triplicate on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. After electrophoresis, the proteins were electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in 2% nonfat dry milk (Bio-Rad, Hercules, CA) for 1 hour at room temperature and then probed with a mouse monoclonal anti- α -SMA at 1:1000 dilution (Sigma), a mouse anti-collagen type I (Sigma) at 1:2000, and a rabbit polyclonal anti-OPG at 1:400 dilution (Santa Cruz Biotechnology), overnight at 4°C. The proteins were detected by enhanced chemiluminescence according to the manufacturer's recommendations (Roche, Indianapolis, IN) and analyzed by densitometry using Gel Pro Analysis software (Media Cybernetics, Silver Spring, MD). The optical densities of the bands were reported as relative density units.

Zymography

MMPs were detected in culture medium by gelatin zymography as described previously.²³ In brief, samples were assayed for protein content using the BCA assay, and all lanes were loaded in triplicate with 12 μ g of protein from each extract alongside with prestained molecular weight standards (Precision Plus Protein Standard; Bio-Rad). After development and staining, density of the clear 68- to 72-kd migrating MMP-2 band on a dark background of stained gelatin was measured using Gel Pro Analysis software (Media Cybernetics, Silver Spring, MD). The sum of optical densities of MMP-2 bands was reported as relative density units.

Alkaline Phosphatase Assay

Cell lysates were analyzed in triplicate for alkaline phosphatase activity using *p*-nitrophenyl phosphate as a substrate and diethanolamine buffer from the Alkaline Phosphatase substrate kit (Pierce). Alkaline phosphatase activity was calculated using a *p*-nitrophenol standard curve and was normalized for total protein content. Alkaline phosphatase activity was also demonstrated in cell culture by staining the cells with 0.5 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate, 5 mg/ml 4-nitro blue tetrazolium, and 5 mmol/L MgCl₂, in 50 mmol/L Tris buffer, pH 9.5 (Sigma). Cells were incubated in the staining solution in the dark for 1 hour at room temperature and then washed.

Osteocalcin Assay

Culture medium samples from each group were analyzed in triplicate for secreted soluble osteocalcin using a rat osteocalcin enzyme-linked immunoassay kit (Biomedical Technologies, Inc., Stoughton, MA), and values were expressed as nanograms per milligram of total protein.

Cbfa-1a Assay

Whole-cell extracts were analyzed for active Cbfa-1 using a TransAM AML-3/Runx2 kit, which combines an enzyme-linked immunosorbent assay (ELISA) format with a specific assay for DNA-binding nuclear transcription factors (Active Motif, Carlsbad, CA). Values were expressed as equivalent micrograms of osteosarcoma nuclear extract (standard provided by Active Motif) per milligram of total protein.

von Kossa Staining

Cells in culture were incubated with 1% silver nitrate solution and placed under UV light for 20 minutes. After several changes of distilled water, the unreacted silver was removed with 5% sodium thiosulfate for 5 minutes, and the cells were rinsed and kept in distilled water. The presence of black stain confirmed the presence of calcium phosphate deposits. The slides were counter-stained with hematoxylin.

Statistical Analysis

Results are expressed as means $\pm$ SEM. Statistical analyses of the data were performed using single-factor analysis of variance. Differences between means were determined using the least significant difference with an α value of 0.05. Asterisks in figures denote statistical significance ($P < 0.05$) for each group compared with controls (cells in medium alone).

Results

Stimulation of α -SMA and SM22 Expression in Fibroblasts

The identification of myofibroblasts was performed through immunofluorescent labeling of α -SMA (Figure 1A). Strong staining for α -SMA was noticed in cells treated with EDPs alone, TGF- β 1 alone, and in EDPs plus TGF- β 1. In addition, fibroblasts treated with EDPs plus TGF- β 1 appeared grouped together in multicellular nodules, where the fluorescent signal was more intense. Analysis of α -SMA mRNA levels indicated a 2.5-fold increase in cells treated with EDPs alone, a fourfold increase in cells treated with TGF- β 1 alone, and an even larger increase for cells treated with EDPs plus TGF- β 1 (Figure 1B). The occurrence of this particular contractile protein in fibroblasts represents a distinctive feature of myofibroblasts.

Figure 1. Expression of α -SMA in fibroblasts exposed to EDPs and TGF- β 1. **A:** Immunofluorescent cell staining for α -SMA. **B:** α -SMA gene expression measured by RT-PCR.

The presence of SM22, a specific SMC marker, was also examined by immunocytochemistry in cells treated with EDPs, TGF- β 1, and both agents together. Its cellular distribution seemed to share a spatiotemporal expression with α -SMA (Figure 2).

Collagen Type I and MMP-2 Up-Regulation

Specific immunofluorescent labeling for collagen type I (Figure 3A) showed an intense signal for the three cell groups treated with EDPs, TGF- β 1, and EDPs plus TGF- β 1. The collagen type I A2 mRNA was approximately 2.5-fold higher in all groups compared with the control (Figure 3B) but without significant differences among the three groups. At the same time, the EDP-stimulated cells secreted large amounts of MMP-2 compared with control and to TGF-treated cells as analyzed by zymography

(Figure 3D). Cells treated with EDPs plus TGF secreted a significantly larger amount of MMP-2 as well. The MMP-2 mRNA displayed the same trend as the protein expression. The increase of collagen type I synthesis along with MMP-2, an enzyme that degrades matrix components, reflects the remodeling tendency of myofibroblasts.

Osteogenic Responses

To appraise the osteogenic changes in cells treated with EDPs and TGF- β 1, the expression of selective bone proteins was assessed. Cbfa1 type II isoform, an osteoblast-specific transcription factor, was slightly elevated at the gene level in the presence of EDPs or TGF- β 1 alone but was significantly ($P < 0.05$) increased in cells stimulated concomitantly with EDPs and TGF- β 1 (Figure 4A). The same pattern was noticed at the protein level, as measured by ELISA (Figure 4B).

Expression of osteocalcin, a protein involved in bone remodeling, was increased approximately 50% at the gene level in the presence of EDPs plus TGF- β 1, compared with EDPs or TGF- β 1 alone (Figure 4C). However, the protein measured by ELISA was mostly higher in response to EDPs, both alone and in combination with TGF- β 1 (Figure 4D). Alkaline phosphatase mRNA was more than 2.5-fold elevated in cells treated with TGF and EDPs plus TGF- β 1 and only 50% elevated in cells treated with EDPs alone, compared with control (Figure 5A). The enzyme activity was significantly higher in the EDPs plus TGF- β 1 group, as measured by colorimetry (Figure 5B) and identified by histochemistry (Figure 5C). Control cells had only faint staining for alkaline phosphatase, whereas staining was more intense in cells treated with EDPs, TGF- β 1, and their combination.

Further evidence that the cells changed their phenotype was the accumulation of calcium in cells maintained

Figure 2. SM22 in fibroblasts exposed to EDPs and TGF- β 1. Immunofluorescent cell staining for SM22.

Figure 3. Modulation of collagen type I and MMP-2 expression in fibroblasts by EDPs and TGF- β 1. **A:** Immunocytochemical staining of collagen type I (red fluorescence). **B:** Collagen type I gene expression measured by RT-PCR. **C:** MMP-2 gene expression measured by RT-PCR (**C**) and MMP-2 enzyme activity measured by gelatin zymography (**D**) (inset) followed by densitometry and expressed as relative density units (RDU).

in culture for 3 weeks and stained with the von Kossa method (Figure 5D). By that time, calcium deposits were noticed in cells treated with EDPs, TGF- β 1, and both agents together but not in control.

Effect of EDPs and TGF- β 1 on OPG Expression

OPG, a possible molecular link between arterial calcification and bone resorption,^{24,25} was also up-regulated in cells treated separately with EDPs or TGF- β 1, as well as

with EDPs plus TGF- β 1, as visualized by immunofluorescence (Figure 6A), analyzed at mRNA level by reverse transcriptase (RT)-PCR and at protein level by Western blot (Figure 6, B and D).

Figure 4. Osteogenic responses in fibroblasts exposed to EDPs and TGF- β 1. **A:** Cbfa-1 gene expression measured by RT-PCR. **B:** Levels of Cbfa-1 protein measured in cell extracts by ELISA. **C:** Osteocalcin (Oc) gene expression measured by RT-PCR. **D:** Protein levels in culture media assayed with an osteocalcin ELISA kit.

Discussion

The composition and structural integrity of the extracellular matrix influence cell phenotypes and, consequently, the development of normal and pathological processes.²⁶ Vascular calcification in the media is characterized by calcium phosphate crystal accumulation within the fragmented elastin lamina²⁷ and the appearance of typical bone proteins secreted by transformed vascular cells.⁶ It was shown that a migratory adventitial cell myofibroblast population, responding to vascular SMC osteopontin production, contributes to vascular remodeling and medial calcification in diabetes and potentially end-stage renal disease.¹⁷ Being α -SMA-positive cells, the vascular cells involved in calcification might be derived from SMCs¹³ or other α -SMA-expressing cells such as pericytes² or myofibroblasts.¹ Pericytes, cells able to express calcified matrix,¹⁸ and myofibroblasts, which could be diverted to the osteoblast lineage,²⁸ share many similarities, and the possible distinction between these cells is not always clear.¹⁷

In studies presented here, we show that in a degraded matrix environment, fibroblasts might become a source of calcifying vascular cells, because they changed into myofibroblasts and osteoblast-like cells in the presence of EDPs. Fibroblasts are considered “sentinel cells” in connective tissue injury and chronic inflammation processes.²⁹ Under these conditions, they modulate into a contractile phe-

notype, for which Prof. Guido Majno et al³⁰ proposed the name myofibroblasts, and contribute to extracellular matrix remodeling and tissue repair.³¹ In our experiments, rat dermal fibroblasts stimulated with soluble EDPs became α -SMA-positive, a valid criterion to define myofibroblasts. They also expressed SM22, a specific smooth muscle marker, suggesting that during their transition, the cells

Figure 6. Expression of OPG in fibroblasts exposed to EDPs and TGF- β 1. **A:** Immunofluorescent cell staining for OPG (red fluorescence). **B:** OPG gene expression measured by RT-PCR. **C:** OPG protein expression measured by Western blotting (inset) followed by densitometry and expressed as relative density units (RDU).

have an intermediate structure, and possibly function, between fibroblasts and SMCs. The α -SMA fluorescent signal was more intense in cells gathin nodules, suggesting that these cells were closer to a smooth muscle cell phenotype than the surrounding ones.

Furthermore, the fibroblasts stimulated with soluble EDPs produced increased amounts of collagen type I and MMP-2, indicating dynamic remodeling activities. MMP-mediated matrix degradation has been linked to vascular calcification. For example, MMP-2 and MMP-9 knockout mice do not show degeneration and calcification after arterial injury,⁷ and site-specific delivery of MMP inhibitors considerably reduced elastin calcification in rats.^{32,33} It is generally perceived that insoluble elastin fibers are highly resistant to proteolysis, because of numerous cross-links and the extreme hydrophobicity of the tropoelastin chains.^{34,35} However, during inflammatory disorders, proteinases secreted from mononuclear neutrophils and macrophages, such as elastase, cathepsin G, and MMPs, may cause significant elastolysis,^{32,36} and soluble peptides are released. These elastin-derived peptides act as matrix-derived cytokines (matrikines) by exhibiting biological activities, such as chemotaxis, protease release, and modulation of cell phenotype, via a 67-kd elastin laminin receptor present on the surface of fibroblasts, smooth muscle cells, and monocytes.^{37–39}

In addition to gaining characteristic properties of myofibroblasts, such as the presence of α -SMA, and increased collagen and MMP-2 synthesis, we noticed that fibroblasts exposed to EDPs for 10 days expressed Cbfa1, a transcription factor essential for osteoblastic differentiation.⁴⁰ Transcription of the Cbfa1/Runx2 gene is driven by two different promoters, denoted P1 and P2, which generate type II and type I isoforms of Cbfa1. The Cbfa1 type I isoform represents a marker of early-stage stromal mesenchymal cells, whereas the type II isoform defines a cell committed to the osteoblast lineage.⁴¹ In our experiment, EDPs induced the expression of Cbfa type II, a marker of osteoblast differentiation. Other bone-specific proteins, such as osteocalcin and alkaline phosphatase, are also overexpressed in treated cells compared with controls. Our results are in agreement with several other studies that present myofibroblasts as cells that normally provide osteoprogenitors for skeleton growth and fracture repair but may also contribute to ossification in valves and arteries by yet ill-defined mechanisms.^{3,6,22,23} In addition, we show that EDP-treated cells maintained in culture for 21 days exhibited calcium deposits associated with multicellular nodule formation.

It is well known that the response to inflammation in bone is osteolysis, whereas in soft tissues, the response is heterotopic calcification, partially accounting for the paradox of arterial mineralization in patients with osteoporosis.⁴² Studies on animal models showed that OPG, probably the long sought-after molecular link between arterial calcification and bone resorption, could inhibit vascular calcification.²⁴ However, it is present in calcified regions of arterial media, probably in an attempt to counteract the pathological process. OPG was expressed by fibroblasts treated with EDPs, suggesting that cell-mediated elastin calcification is controlled by factors typically involved in apparently remote

processes, such as inflammation and bone demineralization, and reveals the complexity of the mechanism.

The osteogenic responses were amplified when, in addition to degraded elastin, fibroblasts were exposed to TGF- β 1. Previous studies have shown that TGF- β 1 promotes calcification of aortic smooth muscle cells in culture^{14,43} and dramatically increases the rate of nodule formation in calcifying vascular cells *in vitro*⁹ and that macrophage-conditioned media enhanced the *in vitro* calcification of vascular cells.⁴⁴ It has also been demonstrated that TGF- β 1 is present within calcified aortic cusps⁴⁵ and mediates the calcification of aortic valve interstitial cells in culture through mechanisms involving apoptosis.⁴⁶ Increased TGF- β 1 expression and signaling are major determinants of the arterial response to injury,⁴⁷ because elastolysis may induce inflammatory reactions and release of active TGF- β 1 from its complex with the latent TGF binding proteins, components of elastin-associated microfibrils.^{48,49} TGF- β 1 generally behaves as an anti-inflammatory and stabilizing factor but also stimulates matrix remodeling, vascular cell osteogenesis, and calcification.⁵ In our experiments, synergistic interactions of EDP-activated fibroblasts with TGF- β 1 may accentuate the effect of elastin peptides or mediate the phenotypic transition toward osteoblast-like cells on a different pathway.

We conclude that elastin degradation products and TGF- β 1, factors typically present in injured cardiovascular matrix environments, promote myofibroblastic and osteogenic phenotypical differentiation in cultured fibroblasts that behave like fibroblast-derived calcifying vascular cells. Our results support the statement that elastin-induced calcification in the arterial wall is associated with vascular cell activation and dynamic remodeling events. Myofibroblasts are crucial in normal tissue repair, where they typically disappear by apoptosis when the tissue integrity is restored. However, their persistence at the site of injury may be associated with collagen accumulation and calcification. The molecular mechanisms underlying ectopic calcium deposition at sites of inflammation in injured tissues remain largely unknown, and our novel data suggest a pathological role for fibroblasts in arterial calcification, regarded as a concluding process of vascular tissue repair.

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