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Original Article

Toward cell therapy for vascular calcification: osteoclast-mediated demineralization of calcified elastin

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Abstract

Background: Elastin-oriented vascular calcification is a clinically significant feature, which involves formation of ectopic bone-like structures. Taking advantage of the similarities between arterial calcification and bone regulation, our hypothesis was that therapeutic approaches for limitation of vascular calcification could be developed using site-specific delivery of autologous osteoclasts. In the present paper, we tested the hypothesis that bone-marrow-derived osteoclasts have the ability to demineralize calcified elastin, without significant alterations in elastin integrity. **Methods:** Active, multinucleated osteoclasts were obtained by in vitro maturation of rat bone-marrow-derived progenitor cells in the presence of vitamin D_3 and retinoic acid. Cell phenotype was validated by staining for tartrate-resistant acid phosphatase, formation of resorption pits on hydroxyapatite-coated disks, and RT-PCR for identification of cathepsin K gene expression. Calcified aortic elastin was seeded with osteoclasts and calcium, and phosphorous levels were monitored in gels and culture media to detect demineralization of elastin. Soluble elastin peptides were also monitored in culture media for elastin degradation. For in vivo experiments, pure aortic elastin was coimplanted with allogenic osteoclasts subdermally into rats, and the degree of elastin calcification and degradation was evaluated using mineral analysis and desmosine quantitation. **Results:** Bone-marrow-derived osteoclasts reduced mineral content of calcified elastin in vitro by 80%. Moreover, in vivo implantation of allogenic osteoclasts in the vicinity of calcifying elastin limited elastin mineralization by almost 50%, in the absence of detectable elastin degradation. **Conclusions:** Osteoclasts have the ability to demineralize calcified elastin, without significant alterations in elastin integrity.

Keywords: Bone marrow stem cells; Differentiation; Gene expression; Resorption

1. Introduction

Vascular calcification occurs during physiological aging and develops in a variety of pathological conditions such as medial arterial elastin calcification, atherosclerosis, calcific aortic stenosis, and Marfan syndrome [1–7]. There are two types of calcification that occur in arteries. One is intimal calcification that occurs mostly with atherosclerotic plaque associated mainly with cells and collagen. The other type of calcification is medial and is associated with elastin. Calcification is clinically recognized as a major risk factor for myocardial infarction, systolic hypertension, heart failure, coronary insufficiency, and possible failure of elastin-containing cardiovascular implants caused by loss of elastic recoil [2,8,9]. Irrespective of the clinical manifestations,

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almost all types of vascular calcifications involve elastin-associated calcification [10–13]. The focus of the present studies is on medial elastin-specific calcification.

In recent years, it has been realized that pathologic arterial calcification is an active remodeling process similar to bone mineralization [14]. In support of this hypothesis, it has been shown that the mineral phase in calcified arteries consists of poorly crystalline hydroxyapatite that resembles physiologic bone mineral [12]. In addition, the presence of newly formed osteoblast-like cells that exhibit up-regulation of bone proteins (such as osteocalcin and osteopontin) [15,16] and osteogenic markers (bone morphogenetic proteins and core-binding factor-protein-1) [17] has been clearly documented. Furthermore, osteoclast-like cells have also been shown to be present in calcified vascular tissue [16]. Bone matrix proteins were also identified in Monckeberg's arterial sclerosis [18], end-stage atherosclerotic plaques, calcified native heart valves, and failed bioprosthetic heart valve implants [19–21].

Taken together, these data indicate that elastin-oriented vascular calcification is a clinically significant feature, which involves formation of ectopic bone-like structures. This pathology progresses with time, is irreversible, and does not spontaneously regress, and there is no therapy to prevent or reverse calcification. Modern imaging techniques are capable of detecting very low levels of calcium deposits in blood vessels [22] and, therefore, may render vascular tissues amenable to nonsurgical treatments for limitation or reversal of progressive aortic calcification.

Taking advantage of the similarities between arterial calcification and bone regulation, our long-term goal is to develop therapeutic approaches for limitation of vascular calcification using minimally invasive, site-specific delivery of autologous osteoclasts. In the present paper, we demonstrate that rat bone-marrow-derived allogenic osteoclasts demineralize calcified elastin in vitro and significantly limit elastin mineralization in a rat subdermal calcification model, without significant alterations in elastin integrity.

2. Materials and methods

2.1. Osteoclast cell culture

Osteoclasts were cultured from adult Sprague–Dawley rat bone marrow as described by Thavarajah et al. [23]. Progenitor cells were isolated from bone marrow aspirates by centrifugation on Ficoll–Paque gradients (Amersham Biosciences, Piscataway, NJ) and cultured at 37°C with 5% CO_2 in Iscove's media, 20% fetal bovine serum, 1% antibiotics/antimycotics, 10^{-6} M all-trans retinoic acid, and 10^{-6} M 1,25-dihydroxyvitamin D_3 (Sigma, St. Louis, MO). Cells were fed every 7 days. Under these conditions, bone marrow progenitor cells differentiate into mature osteoclasts after approximately 14 days.

2.2. Validation of osteoclast phenotype and biological function

Mature osteoclasts were stained with hematoxylin and eosin (H&E) after a cytospin procedure, as well as for activity of tartrate-resistant acid phosphatase (TRAP) in culture according to manufacturer's instructions (Sigma). For validation of mineral-resorptive abilities, hydroxyapatite-coated quartz discs (osteologic discs, BD Biosciences, Franklin Lakes, NJ) were seeded with rat osteoclasts, and after 7 and 14 days in culture, discs were bleached to remove cells, stained for hydroxyapatite using the von Kossa technique, and observed under normal light microscopy.

2.3. Preparation of pure porcine aortic elastin

Porcine hearts were obtained from a local slaughterhouse and transported to the laboratory on ice. A supravalvular aortic segment (3–4 cm) was dissected, cleaned to remove fat and adherent tissues, and rinsed in cold saline. Aortic elastin was purified as previously described [24,25]. Briefly, 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 via BCA assay (Pierce, Rockford, IL). The washed aorta was autoclaved four times in distilled water and washed with distilled water until no protein was detected in the solution via BCA assay. This was then followed by defatting the aorta with ethanol and diethyl ether and lyophilization. This procedure extracts all cellular materials, as well as collagenous and noncollagenous components, leaving pure elastin intact.

2.4. In vitro study: osteoclast-mediated demineralization of calcified elastin

Purified elastin was subdermally implanted in juvenile Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) as described previously by our group [25]. Elastin implants were aseptically retrieved after 7 days, lyophilized, homogenized to a fine powder, and incorporated in a pH 7 neutralized soluble collagen solution (3 mg/ml Vitrogen, Cohesion Technologies Inc., North Bend, WA) on ice at a final concentration of 2.5 mg calcified elastin/ml collagen. The mixture was added to 24-well cell culture plates (0.5 ml gel/well) and incubated at 37°C with 5% CO_2 for 30 min to solidify. Osteoclasts and rat fibroblasts were then added to wells at a concentration of 5×10^4 cells per gel (n=4 per group). Media were replaced at 7 days and stored for calcium and elastin peptide analysis. At Day 14, all media and gels were removed and stored for analysis. Control groups consisted of gels with and without osteoclasts (no elastin) and elastin in collagen gels without osteoclasts (n=4 per group).

Calcium and phosphorous content of gels and media was determined via atomic absorption spectrophotometry and a molybdate complexation assay, respectively, as described

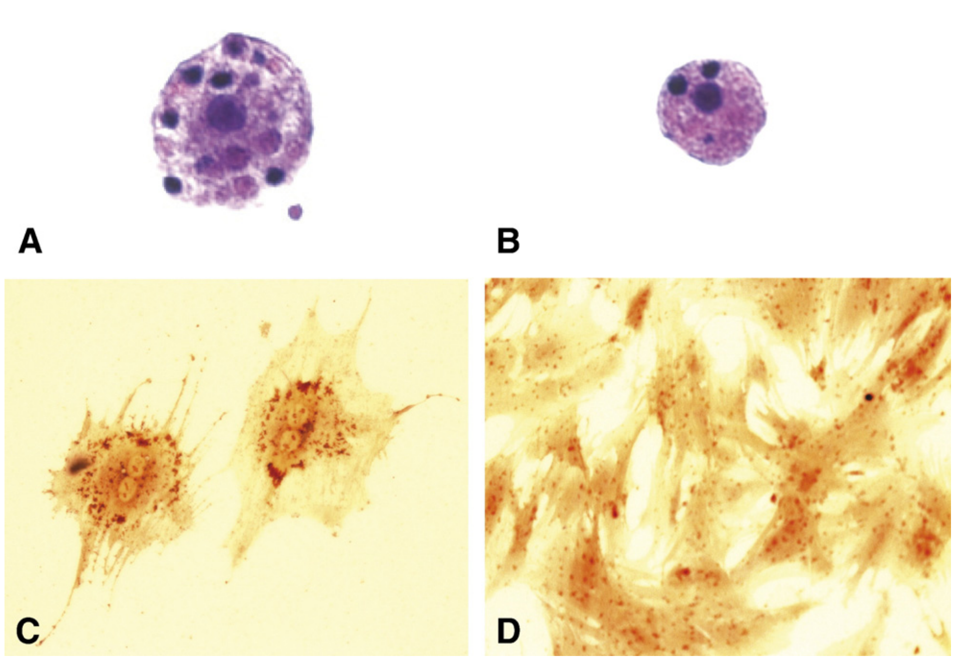

Fig. 1. Histological and cytochemical characterization of bone-marrow-derived, in vitro matured rat osteoclasts. H&E staining shows multinucleated cells (A, B), and cytoplasmic TRAP staining (reaction product is reddish-brown) reveals a majority of positive cells (C, D). Note that cells shown in Panels A and B are suspended cells obtained by cytospin (round morphology) and that cells shown in Panels C and D are attached to the culture plate (spreading). Original magnifications, ×200.

before [26]. Calcium and phosphorous values are expressed as micrograms per milligram of dry weight for gels and as micrograms per milliliter for culture media. All assays were done in triplicate.

Quantitation of soluble elastin peptides was performed in culture media via a competitive enzyme-linked immunosorbent assay (ELISA) as described by Wei et al. [27] with minor modifications [25]. Briefly, media samples were

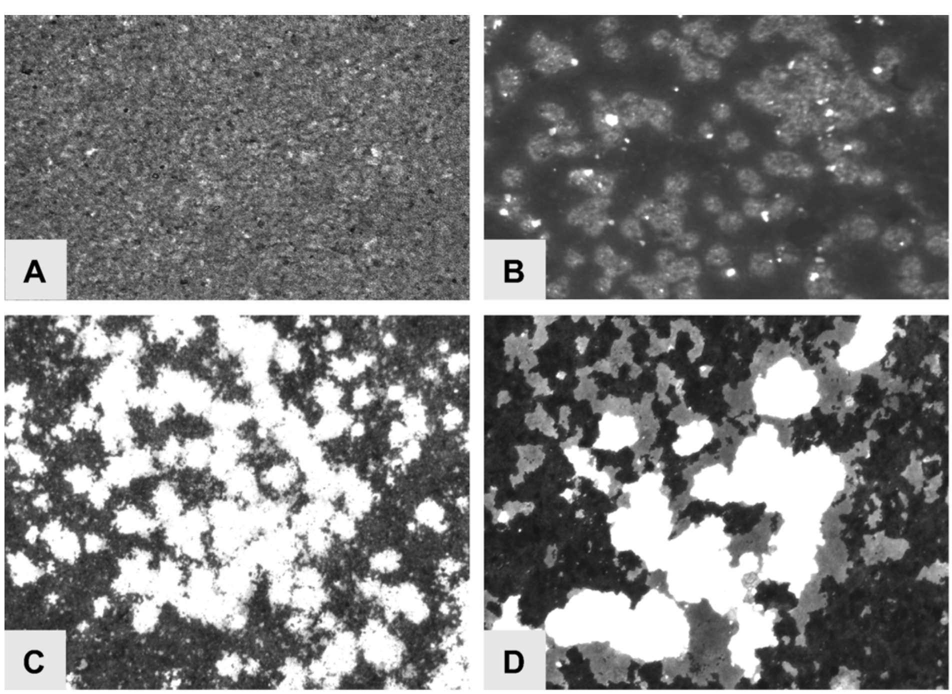

Fig. 2. Demonstration of the mineral-resorptive capacity of rat osteoclasts. Bone-marrow-derived, in vitro matured osteoclasts were cultured on hydroxyapatite-coated glass discs and analyzed for formation of resorption pits after 7 days (B) and 14 days (C, D) by von Kossa staining (hydroxyapatite stains, dark gray). Control discs, cultured in the absence of cells are shown in Panel (A). Resorption pits appear as light gray spots after 7 days due to partial mineral removal (B), while after 14 days, complete mineral dissolution yields white spots upon staining (C, D). Original magnifications, ×200.

mixed with rabbit antielastin antibody (Elastin Products Company Inc., Owensville, MO) and added to wells of microtiter plates that have been precoated with elastin peptides (Elastin Products Company Inc.). Detection was completed using goat antirabbit IgG-peroxidase conjugate and H_2O_2/*ortho*-phenylene diamine hydrochloride. All assays were done in triplicate, and concentrations of soluble elastin peptides were expressed as micrograms per milliliter of medium.

2.5. In vivo study: osteoclast-mediated limitation of elastin calcification

To monitor cell distribution after implantation, we tagged osteoclasts with Cell Trace Far Red DDAO-SE fluorescent tag (DDAO-SE), a fixable, far-red-fluorescent tracer for long-term cell labeling, according to the supplier's instructions (Molecular Probes, Eugene, OR). Purified aortic elastin was dispersed in cold, neutralized acid soluble collagen, mixed with 2.1×10^4 tagged rat osteoclasts, and after overnight solidification, gels were implanted subdermally via a small skin incision in juvenile male Sprague–Dawley rats (Harlan Laboratories). Cell viability was not affected by this procedure (data not shown). The control groups were elastin in collagen gel alone and osteoclasts in collagen alone (n=3 per group). Rats were humanely euthanized after 1, 3, and 7 days; the implants were retrieved and analyzed for mineral content, histology, and gene expression. Elastin content was assayed by desmosine analysis using a radioimmunoassay [28].

All animal studies were performed according to protocols approved by the Clemson University Animal Research Committee. Animal care was provided by trained veterinarians according to NIH guidelines for the care and use of laboratory animals (NIH publication No. 86-23, revised 1985).

2.6. Analysis of gene expression

RT-PCR identification of cathepsin K, an osteoclast-specific gene, was done essentially as described before [25]. Briefly, 400 ng of high-purity total RNA extracted from each explant (n=3 per group) was reverse transcribed, and PCR was performed for the cathepsin K gene (accession # NM_031560) using the following primers: 5′-CACGGGAAGCAGTACAACAG-′3 (forward) and 5′-AGTCATCTTCTGAACCACTTCTTC-3′ (reverse). For β2-microglobulin (β2-MG), accession #NM_012512, primers were 5′-CGTGATCTTTCTGGTGCTTGTC-3′ (forward) and 5′-ACGTAGCAGTTGAGGAAGTTGG-3′ (reverse). Cathepsin K gene expression was normalized to the expression of β2-MG as a housekeeping gene and compared with control samples (subdermally implanted elastin/collagen gels), using the $2^{-\Delta\Delta}$CT method [29].

Results are expressed as means±S.E.M. Statistical analyses of the data were performed using single-factor

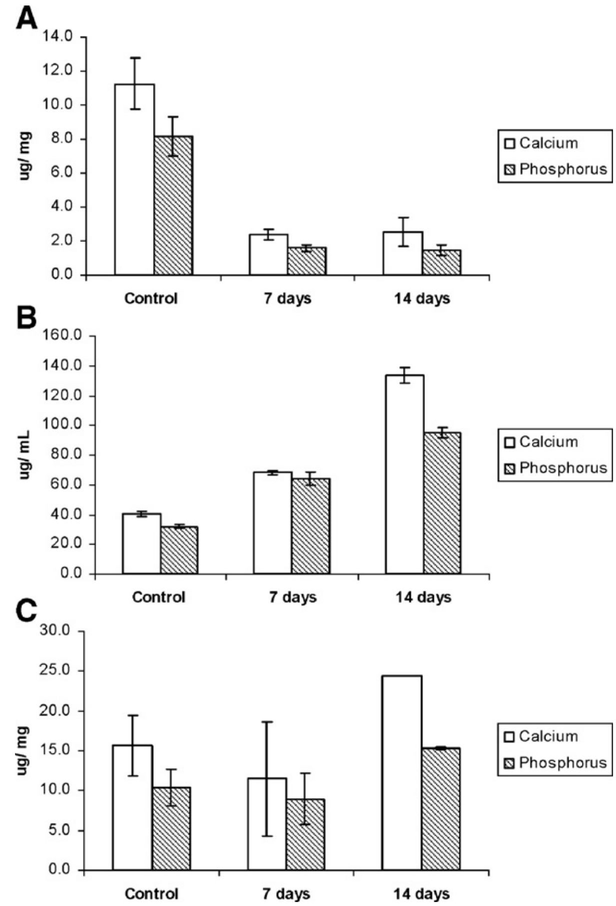

Fig. 3. Osteoclast-mediated demineralization of calcified elastin in vitro. Calcium and phosphorus content of calcified elastin (in collagen gel) cultured with bone-marrow-derived, in vitro matured osteoclasts (A), media from the same culture (B), and calcified elastin cultured with rat skin fibroblasts (C). Controls consisted of collagen gels containing calcified elastin (EL only) incubated in media with no cells. Calcium levels for elastin samples with osteoclasts were significantly lower with corresponding increase in calcium in the media (P<.05) for both gels and culture media, while culturing with fibroblast did not resorb calcium from calcified elastin (P>.05).

analysis of variance. Subsequently, differences between means were determined using the least significant difference with an alpha value of .05.

3. Results

Several tests were performed in order to validate cell phenotype in our culture system after osteoclast maturation. H&E staining showed the presence of very large cells possessing multiple nuclei (Fig. 1A and B). The cells display a rounded morphology due to the cytospin procedure for cell attachment to slides. Osteoclasts in culture were also positively identified by TRAP staining as evidenced by the reddish-brown intracytoplasmic stain (Fig. 1C and D). PCR analysis of cathepsin K gene expression further confirmed that the bone-marrow-derived, in vitro matured cells were osteoclasts (results not shown).

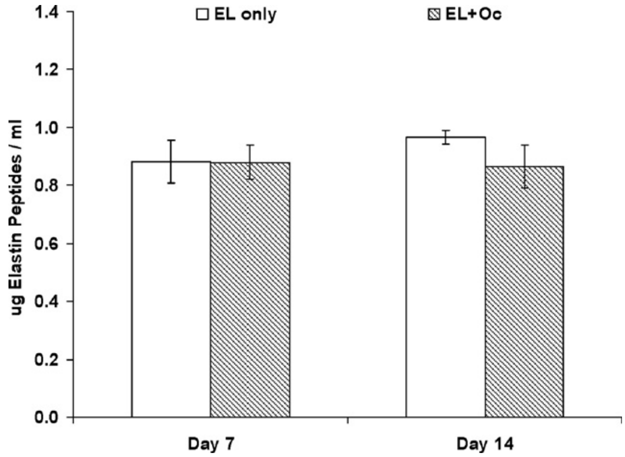

Fig. 4. Soluble elastin peptide assay for evaluation of elastin degradation during demineralization by osteoclasts. Bone-marrow-derived, in vitro matured osteoclasts were cultured on collagen gels containing calcified vascular elastin (EL+Oc), and the culture media was assayed for the presence of degraded elastin by an elastin peptide ELISA after 7 and 14 days. Controls consisted of collagen gels containing calcified elastin (EL only). Values are expressed as micrograms of elastin peptide per milliliter of culture media. Differences were not statistically significant (P>.5).

For validation of mineral-resorptive abilities, we seeded osteoclasts onto hydroxyapatite-coated glass discs, which simulate the mineral phase of bone and allow visualization of resorption activity. After 7 days in culture, resorption pits were clearly visible on the discs (Fig. 2B). The light gray tint of the resorption pits indicated partial hydroxyapatite dissolution. After 14 days in culture, larger numbers of complete and more confluent resorption pits (white) were observed (Fig. 2C and D). These results further verify that our technique for obtaining osteoclasts from rat bone marrow was successful because the mature osteoclasts are capable of hydroxyapatite dissolution, even in the absence of extracellular matrix substrates. This biological activity is highly specific for osteoclasts because rat skin fibroblasts, when seeded on hydroxyapatite discs in the same experimental conditions, failed to produce resorption pits (results not shown).

The ability of osteoclasts to demineralize calcified elastin was investigated by incorporating calcified elastin into a collagen gel and seeding osteoclasts on top of the gel. Calcium and phosphorus analysis showed a significant (P<.05) reduction of mineralization of calcified elastin in the presence of osteoclast (Fig. 3A). This was accompanied by a significant (P<.05) increase in calcium content of cell culture media collected from the same samples (Fig. 3B). This activity was specific for osteoclasts because rat skin fibroblasts, when seeded on calcified elastin/collagen gels, failed to reduce elastin mineralization (Fig. 3C).

To check for potential elastin degradation during in vitro demineralization of calcified elastin, we also performed an elastin peptide assay on culture media. Results showed no significant release of soluble elastin degradation products after 7 and 14 days in the presence of osteoclasts (P>.5; Fig. 4) as compared with control implants. Also, no significant amount of degradation products was observed when the amount of cells was doubled (results not shown).

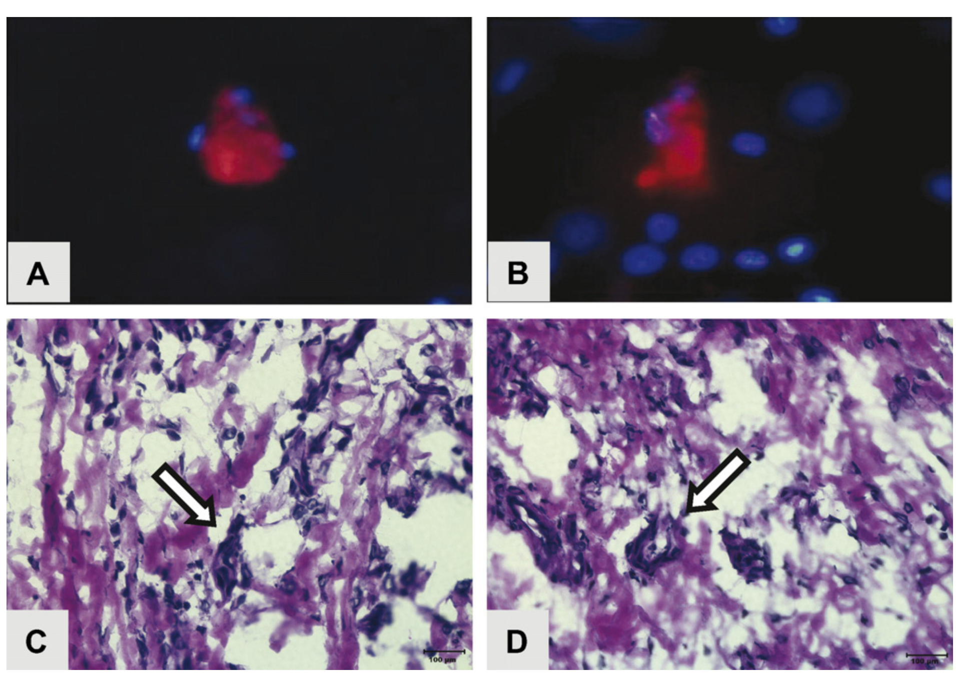

Fig. 5. Rat subdermal implantation of pure elastin with allogenic osteoclasts. Osteoclasts were fluorescently labeled before implantation with DDAO-SE (red fluorescence) and were identified in the implants at Days 1 and 3 (A, B). Nuclei were stained with DAPI (blue fluorescence) and digitally superimposed. H&E staining of explants also verified the presence of multinucleated osteoclasts at Day 3 (C, D, arrows).

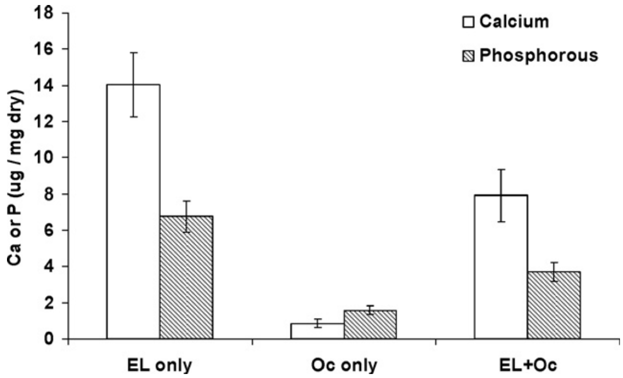

Fig. 6. Allogenic osteoclasts limit elastin calcification in the subdermal implantation model. Fluorescently labeled osteoclasts were coimplanted with elastin in collagen gels, and 7-day explants were analyzed for calcium (Ca) and phosphorus (P) content. Controls consisted of collagen gels containing osteoclasts (Oc only). Values are depicted as micrograms of Ca or P per milligram of dry explant. Differences between calcium or phosphorous levels for elastin samples with osteoclasts (EL+Oc) and without (EL only) were statistically significant (P<.05).

In a second series of experiments, in order to test for the ability of osteoclasts to limit or reverse elastin calcification, we coimplanted pure aortic elastin subdermally with allogenic osteoclasts. The osteoclasts were fluorescently labeled before implantation with DDAO-SE (red fluorescence) for monitoring purposes. Tagging did not alter cell viability or their ability to induce formation of resorption pits when cultured on hydroxyapatite-coated discs (not shown). Fluorescent osteoclasts were identified in the implants at Days 1 and 3 (Fig. 5A and B) but not at 7 days after implantation. H&E staining also verified the presence of multinucleated osteoclasts at Day 3 (Fig. 5C and D). At Day 7, the implants were removed and analyzed for calcium and phosphorus content. In the presence of osteoclasts, elastin mineralized to about half the extent as it did without osteoclasts (Fig. 6). Alizarin red staining for calcium visually verifies the results of the calcium analysis (Fig. 7). Implants from all time points were also analyzed for cathepsin K gene expression by RT-PCR to verify the presence and functional status of osteoclasts. Samples explanted at 3 days exhibited more than twofold up-regulated expression of the cathepsin K gene (2.4±0.9-fold) as compared with controls, while 7-day explants expressed unremarkable levels (0.7±0.3-fold) as compared with controls. Desmosine analysis of 7-day explants revealed no significant in vivo elastin degradation in the presence of osteoclasts (5.7±0.9 and 6.0±0.6 nmol desmosine/mg protein for elastin with osteoclasts and elastin without osteoclasts, respectively, P>.8).

4. Discussion

Vascular calcification shares many similarities to bone formation. Fundamentally, the mineral phase in calcified arteries is composed of poorly crystalline hydroxyapatite that resembles bone mineral. Osteoblastic and osteoclastic cells populate calcified arteries and bone-specific proteins, and cytokines have been found in clinically removed calcified specimens. While collagen is the main organic component in bone, calcium deposits in pathologic arteries associate mainly with elastin.

Bone homeostasis and control of physiological bone composition are continuous remodeling processes dependent on bone formation by osteoblasts and bone resorption by osteoclasts [30]. In bone pathology, reduction in bone

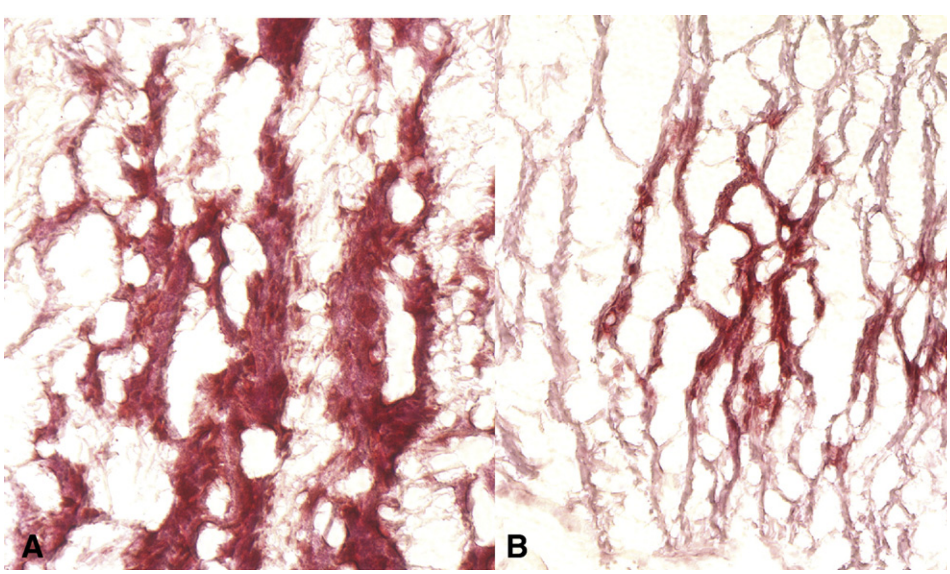

Fig. 7. Histological analysis of elastin calcification. Alizarin red stains calcified matrix red. Subdermally implanted elastin alone (A) calcified ~50% more than subdermally implanted elastin with osteoclasts (B). Magnification, ×400.

mineral formation, similar to that found in osteoporosis, is mainly associated with excessive activities of osteoclasts, which overtake the bone-formation capacity of osteoblasts. Although both osteoblastic and osteoclastic cells are present in calcified arteries, it is apparent that the microenvironment is inductive for progressive, uncontrolled mineralization. Our hypothesis was that altering the balance of bone formation versus bone resorption by site-specific delivery of autologous osteoclasts would facilitate limitation of arterial calcification. Since elastin is one of the major calcification substrates in arteries, we focused our studies on osteoclast-mediated reversal of elastin calcification.

Osteoclasts are cells of hematopoietic descent, and their precursors can be differentiated into osteoclasts in vitro under specific maturation conditions. In our studies, we collected bone marrow progenitor cells from adult rats and cultured them in the presence of vitamin D_3 and retinoic acid. These cells were positive for TRAP activity, which further validated that they were osteoclasts. Osteoclasts were further characterized by seeding on hydroxyapatite-coated glass discs and culturing them for up to 14 days. Hydroxyapatite staining of these discs revealed numerous resorption pits at 14 days. This in vitro activity assay provided definitive proof of the mineral-resorptive capacities of our bone-marrow-derived osteoclasts [31], even in the absence of extracellular matrix.

To evaluate the ability of osteoclasts to demineralize calcified vascular elastin, we first implanted purified aortic elastin subdermally in juvenile rats and explanted samples after 7 days. We have previously used this accelerated model to investigate fundamental mechanisms of elastin calcification and have shown that calcium deposits are exclusively associated with the elastin fibers [26]. Typically, elastin calcifies in 7 days to about 30–40% of its maximum mineralization capacity in this model. Calcified elastin was incorporated into a Type I collagen gel, and mature osteoclasts were seeded on top of that gel. Mineral analysis showed an 80% reduction of elastin calcification levels in the presence of osteoclasts ($P<.05$). This was accompanied by a more than 70% increase in calcium content of cell culture media collected from the same samples ($P<05$). The molar ratios of calcium to phosphate (mean of about 1.2) revealed insignificant differences between groups ($P>0.5$), suggesting that both calcium and phosphate ions have been concomitantly removed during osteoclast-mediated demineralization of calcified elastin.

These results indicate that osteoclasts have the ability to resorb mineral by its dissolution from calcified elastin, similar to the way they resorb bone in physiological conditions. This process appears to be specific to osteoclasts because rat skin fibroblasts, when seeded on calcified elastin/collagen gels, failed to induce demineralization.

During normal bone resorption, osteoclasts secrete acid that dissolves the mineral phase and proteases that degrade the organic matrix of bone. In order to test whether osteoclasts degraded elastin in the process of in vitro demineralization, we used ELISA to measure amounts of soluble elastin peptides released by osteoclasts in culture media retrieved from the gels. Results showed no significant differences between elastin peptide levels in controls (elastin alone) as compared with elastin incubated in the presence of osteoclasts. Taken together, these results indicate that bone-marrow-derived rat osteoclasts have the capacity to demineralize calcified elastin in vitro, without degrading the elastin substrate. However, the detailed mechanisms of osteoclast-mediated demineralization of calcified elastin are not clear and require further fundamental studies.

In order to evaluate the ability of allogenic osteoclasts to limit elastin calcification in vivo, pure aortic elastin was coimplanted subdermally into rats together with bone-marrow-derived, in vitro matured, fluorescently labeled allogenic osteoclasts. We have previously shown that elastin mineralization in this model shares many similarities with bone formation, including the presence of osteoblastic cells that are positive for core-binding factor alpha surrounding calcified elastin [25]. The osteoclasts could be identified in elastin implants after 3 days via fluorescence microscopy, histology, and PCR, clearly indicating that live, functional osteoclasts were present in the vicinity of implanted elastin. However, at 7 days, no osteoclasts were evident, confirming that osteoclasts have a limited life span after implantation [32]. Calcium and phosphorus analysis revealed that in this experimental model, the presence of osteoclasts in the vicinity of elastin implants limited deposition of elastin-specific calcification.

To the best of our knowledge, this is the first published attempt to use cell therapy for limitation of elastin calcification. Since osteoclasts are the only cells capable of resorbing bone [33], this approach seems reasonable. However, at this point, the precise mechanisms involved in osteoclast-mediated limitation of elastin calcification in this experimental model are not ascertained. We have shown recently that calcification of elastin in this subdermal implantation model is accompanied by remodeling and osteogenic events, which closely mimic those involved in bone formation [25]. Notably, mineralization occurs on a different biochemical substrate, that is, elastin fibers, as compared with Type I collagen fibers normally present in bone. In addition, elastin mineralization occurs in this model at a significantly accelerated rate, with the first calcium deposits being detectable as early as 24 h after implantation [26]. Based on our results, we hypothesize that quickly developing mineral deposits on the surface of elastin fibers prompted attachment of osteoclasts to those deposits and resulted in dissolution of some of the hydroxyapatite salts. It is also possible that osteoclasts secreted mineralization inhibitors such as pyrophosphates or bone-specific proteins that could have influenced mineralization of elastin. Such questions could be answered by allowing elastin to mineralize to a certain extent prior to delivery of osteoclasts. Such studies are underway to gain a detailed understanding of these interactions.

In conclusion, bone-marrow-derived osteoclasts have the ability to directly resorb mineral by its dissolution from calcified elastin, without significant degradation of the elastin substrate. Moreover, in vivo implantation of allogenic osteoclasts in the vicinity of calcifying elastin limits elastin mineralization. Since osteoclasts can effectively exert their demineralization functions outside of their bone environment, our results provide exciting opportunities for cell-mediated therapy of vascular calcification.

5. Summary

Elastin-associated vascular calcification involves formation of bone-like structures. We hypothesized that osteoclasts, the only cells capable of remodeling bone, could be utilized to control vascular calcification. We demonstrate that bone-marrow-derived, in vitro matured allogenic osteoclasts have the ability to interact with calcified aortic elastin and reduce its mineral content in the absence of detectable elastin degradation.

Acknowledgment

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The reader is referred to the web version of this article (www.cardiovascularpathology.com) for color reproduction of Figs. 1, 5, and 7.

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