Decorin Promotes Aortic Smooth Muscle Cell Calcification and Colocalizes to Calcified Regions in Human Atherosclerotic Lesions

Jens W. Fischer, Susie A. Steitz, Pamela Y. Johnson, Allen Burke, Frank Kolodgie, Renu Virmani, Cecilia Giachelli, Thomas N. Wight

Objective—Ectopic calcification localized to the intima of atherosclerotic plaque is a risk marker for cardiovascular events and increases the risk of aortic dissection during angioplasty. A variety of extracellular matrix molecules such as collagen type 1, bone sialoprotein, and osteopontin are known to regulate the biomineralization of bone and ectopic vascular calcification. In the present study, it was investigated whether decorin, a small leucine-rich proteoglycan expressed in bone and atherosclerotic plaque, is involved in arterial calcification.

Methods and Results—Calcification was induced in cultured bovine aortic smooth muscle cell (BASMC) by the addition of β-glycerophosphate or inorganic phosphate. Northern and Western analysis revealed that decorin expression was strongly upregulated in mineralizing BASMC. Furthermore, overexpression of decorin using a retroviral expression vector resulted in a 3- to 4-fold elevation of calcium deposited on the BASMC monolayer. Increased calcification in response to decorin could also be mimicked by adding exogenous decorin to the cultures. In addition, human coronary atherosclerotic lesions taken from sudden-death patients showed marked colocalization of calcium deposits with decorin.

Conclusions—Decorin induces calcification of arterial smooth muscle cell cultures and colocalizes to mineral deposition in human atherosclerotic plaque, suggesting that decorin functions as promoter of intimal calcification. (Arterioscler Thromb Vasc Biol. 2004;24:2391-2396.)

Key Words: small leucine rich proteoglycan • decorin • extracellular matrix • vascular calcification • atherosclerosis
and bovine SMC\textsuperscript{11,17,18} support the suggestion that mesenchymal-derived vascular cells participate in the regulation of mineral deposition by mimicking bone formation.

The small leucine-rich proteoglycan (SLRP), decorin,\textsuperscript{19} colocalizes with areas of active bone calcification. Decorin is composed of a 38 000-dalton M, core protein containing one dermatan sulfate or chondroitin sulfate chain, and is expressed in skeletal tissues such as developing and adult bone and most collagen-rich mesenchymal tissues, such as the adventitia of blood vessels and the skin.\textsuperscript{19,20} Defined functions of decorin include regulation of transforming growth factor-\(\beta\) activity,\textsuperscript{21} control of tumor cell proliferation,\textsuperscript{22} and regulation of collagen fibrillogenesis.\textsuperscript{19,23} Furthermore, decorin binds hydroxyapatite\textsuperscript{24} and is localized to the newly developing fetal bone matrix at all stages of bone formation and within the matrix of resting nonarticular cartilage.\textsuperscript{20}

In cultured aortic SMC, decorin was shown to regulate transforming growth factor-\(\beta\) activity\textsuperscript{25} and collagen gel contraction, as well as collagen type I expression.\textsuperscript{26} During atherosclerosis and restenosis, extracellular decorin associates with SMC and collagen type I and III in the fibrous cap and with SMC and macrophages in the core region.\textsuperscript{27,28}

In the present study, we have investigated the effect of elevated decorin expression in arterial calcification using a bovine aortic smooth muscle cell (BASMCC) culture model. This model has previously been characterized with respect to its relevance to in vivo arterial calcification including matrix vesicle formation, apatitic mineral deposition, and induction of calcification-related proteins such as osteopontin and alkaline phosphatase.\textsuperscript{18} In addition, the topographical relationship between calcified areas and decorin deposition in the intima of human atherosclerotic lesions was assessed.

### Methods

#### Cell Culture

Primary BASMC from the media were prepared by the explant method as described by Wada et al\textsuperscript{18} and propagated in Growth Medium (DMEM containing 4.5 g/L glucose, 15% fetal bovine serum, 10 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\). Purified bovine decorin that was prepared by dissociative extraction from bovine tendons was used to study the function of exogenously added decorin (0.05 and 0.5 \(\mu\)g/mL). All other reagents were from Sigma unless stated otherwise.

#### Construction of Bovine Decorin Retrovirus and Stable Transfection of BASMC

The complete coding sequence of bovine decorin\textsuperscript{29} (clone PG28) was cloned into the retroviral LXSN vector. Subsequently, the vector containing bovine decorin (LDSN) was used to generate pools of BASMC overexpressing bovine decorin as described previously.\textsuperscript{30} BASMC infected with the empty control vector (LXSN) served as controls.

#### BASMC Calcification and Quantitation

BASMC were plated on tissue culture plastic at 10 000 cells/cm\(^2\). On confluence, BASMC monolayers were treated with either growth medium or calcification medium (growth medium containing 10 mmol/L \(\beta\)-glycerophosphate or 2.4 mmol/L inorganic phosphate [Pi]) for a maximum of 14 days.\textsuperscript{15,16} Every 3 days, the medium was replaced with either fresh growth medium or calcification medium. Calcification was visualized by von Kossa staining and quantitated as indicated previously.\textsuperscript{18} Briefly, at the indicated times, culture media were removed, monolayers washed with phosphate-buffered saline, and the mineral dissolved using 0.6 N HCl overnight at 37°C. At this time, HCL was collected and cell lysates prepared in 0.1 N NaOH containing 0.1% SDS. Calcium content of the HCL solution was determined using the o-cresolphthalein complexone method (Sigma Diagnostics) as directed by the manufacturer. Protein concentration of the HCL solution was determined using the BCA Assay (Pierce). Calcium content was normalized to protein concentration. Calcium deposition was visualized by von Kossa staining (30 minutes, 5% silver nitrate).

#### Detection of Decorin Core Protein

Equal volumes of conditioned (24 hours) tissue culture medium of LDSN-transfected and LXSN-transfected, as well as of untransfected BASMCs, were used to detect synthesis of decorin by immunoblotting (anti-bovine decorin antibody, LF-94) as described previously.\textsuperscript{30} Cell layers from BASMC cultured in the presence or absence of calcification media were extracted with 8 mol/L urea, 2 mmol/L EDTA, 25 mmol/L NaCl, 0.5% Triton X-100, and 50 mmol/L Tris HCl, pH 7.5. Equal volumes of DEAE-treated extracts were digested with Chondroitinase ABC and used to detect decorin by immunoblotting (anti-bovine decorin antibody, LF-94) as previously described.\textsuperscript{30}

BASMC were cultured 10 days after confluence in the presence or absence of calcification media. On day 10, cells were fixed in 2% neutral buffered formalin. Decorin was localized with the use of LF-94 and a fluorescein isothiocyanate-tagged secondary antibody.

#### Northern Analysis

Total RNA was isolated from BASMC cultures grown in the presence of either growth or calcification medium by use of TRI-Reagent (Molecular Research Center). Subsequently, Northern analysis was performed using the full-length bovine decorin cDNA (PG28) and full-length human biglycan cDNA (P-16) as described previously.\textsuperscript{30}

#### Morphological Analysis of Human Atherosclerotic Plaque

Twelve atherosclerotic lesions from sudden-death patients were identified. Coronary segments were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections cut at 6-\(\mu\)m thickness were mounted on charged slides and stained for calcium with the von Kossa stain or for decorin with rabbit polyclonal antibody (Anti-bovine decorin antibody, LF-94) as described previously.\textsuperscript{30} The reactions were visualized by using streptavidin conjugated to horseradish peroxidase according to a method described previously. Color development with diamobenzidine was contrast-enhanced with NiCl\(_2\).

#### Statistical Analysis

Data are the mean±SEM of independent experiments. Statistical analysis was performed by 1-way ANOVA and the means were considered significantly different if \(P<0.05\). Subsequently, Dunnett multiple comparison test or comparison of selected pairs (Bonferroni) was performed, and \(P<0.05\) was considered significant.

#### Results

Upregulation of Decorin in Calcifying BASMC

Mineral deposits were detectable by light microscopy within 7 days in the presence of the organic phosphate donor \(\beta\)-glycerophosphate (10 mmol/L) or P\(_2\) (2.4 mmol/L) and persisted throughout the culture period up to 14 days (Figure 1). No mineral was observed in cultures grown for the same period of time in the absence of an exogenous phosphate source.

The expression of the chondroitin/dermatan sulfate proteoglycans, decorin, biglycan, and versican was analyzed by Northern blotting in 3 experiments in arterial SMCs cultured 4, 7, 10, and 14 days in normal growth medium versus calcifying medium.
The mean fold difference of biglycan mRNA expression slightly decreased over this time period in the presence of \(\beta\)-glycerophosphate (Figure 2A), whereas versican mRNA expression was unchanged (data not shown). In contrast, decorin mRNA expression was significantly increased in the presence of \(\beta\)-glycerophosphate, peaking at 7 and 10 days. Subsequently, decorin expression decreased but remained elevated at day 14 when compared with control cells (Figure 2B).

To determine whether the changes in decorin mRNA were translated into differences in the synthesis of decorin core protein, Western blot analysis was performed. Accumulation of decorin core protein in the conditioned media was dramatic by 10 days of treatment (Figure 3A). Furthermore, cell-associated decorin was also increased under calcifying conditions, as shown by Western blot and immunocytochemistry (Figure 3B and 3C).

**Retroviral Overexpression of Decorin Enhances Calcification of BASMC**

Stable overexpression of bovine decorin in BASMC was achieved by a retroviral approach. After infection with the LDSN virus, cell pools were selected by G418 and bovine decorin secreted into the cell culture medium analyzed by Western blotting. As shown in Figure 4A, a dramatic increase in decorin secretion into the cell culture medium was achieved. To investigate the role of decorin in calcification of BASMC, the retrovirally transduced cells were cultured over the course of 14 days under normal growth conditions versus calcifying conditions. Calcium content was significantly increased in LDSN cells versus LXSN cells at 10 days (Figure 4B). After 14 days, the increase in mineralization of LDSN had increased to >3- to 4-fold of that of LXSN cells. In contrast, under normal growth conditions, no difference in calcification was observed in LXSN versus LDSN cells.

**Addition of Purified Bovine Decorin Promotes Calcification of BASMC**

To determine whether addition of exogenous decorin would influence the calcification of arterial SMCs, decorin from bovine tendon was added to the cultures. Subsequently, every 3 days for a total of 10 days, the BASMC monolayer media was replaced with growth medium, calcification medium alone, or calcification medium containing purified decorin at the concentrations indicated (Figure 5). After 10 days, mineralization was increased \(\approx 2\)-fold over vehicle-treated controls by addition of 0.05 and 0.5 \(\mu\)g/mL purified decorin.

**Decorin Colocalizes With Calcified Areas in Human Atherosclerotic Lesions**

A sampling of human atherosclerotic lesions from patients undergoing sudden death indicates that these lesions contain pockets of calcium identified with the von Kossa stain (Figure 6) within the lesion. These regions were frequently found as discrete loci within an expanded intimal region of the atherosclerotic plaque (Figure 6A, 6C, and 6E). Using near-adjacent sections, virtually every area that stained positive for calcium was positive for decorin immunostaining (Figure 6B, 6D, and 6F). There was little if any decorin immunostaining in the noncalcified zones in the atherosclerotic lesions.

**Discussion**

Dystrophic arterial calcification in the intima of atherosclerotic plaques increases the risk of vessel fracture during angioplasty and is associated with increased risk of cardiovascular events.\(^5\) The known factors regulating vascular calcification are in part identical with those known to be involved in bone mineralization such as alkaline phosphatase, bone morphogenic protein-2, and ECM molecules such as collagen type 1, osteopontin, osteonectin, and osteocalcin.\(^10,12\)
However, it is not known whether other ECM molecules known to be involved in bone mineralization such as decorin and biglycan play a role in vascular calcification. Mixtures of decorin and biglycan isolated from bone increased hydroxyapatite formation in mineralizing collagen gels. Decorin and biglycan are required for normal bone formation. For example, biglycan-deficient mice exhibit reduced peak bone mass and an osteoporotic phenotype, whereas the bone structure of decorin-deficient mice is normal. The double-knockout mice for decorin and biglycan have an enhanced osteoporotic phenotype that develops even earlier compared with the biglycan single-deficient mice, suggesting that decorin and biglycan are both required for normal bone development, and that biglycan compensates for the absence of decorin in decorin knockout mice. We now report that decorin overexpression enhances calcification of tissues other than bone, such as sheets of arterial SMCs, and colocalizes to regions of atherosclerotic plaques in arteries involved in calcification.

In calcifying BASMC, a transition to an osteogenic phenotype occurs as described earlier. Increased cbfa-1 expression at day 3 after addition of β-glycerophosphate is the first detectable change associated with calcification. This is followed by down-regulation of protein expression of SMrα-actin and SM22α at day 7 and day 10, respectively. Alkaline phosphatase is strongly increased at day 10 in this model. The present results show that increased decorin expression (days 7 to 14) occurs simultaneously to the phenotypic transition of calcifying BASMC and coincides with the first detectable presence of mineral deposition in this model. The increase in decorin expression on mineral deposition is similar to findings reported by others studying changes in decorin levels during bone matrix mineralization. For example, it may be that increased cbfa-1 expression and cbfa-1–dependent gene expression in response to β-glycerophosphate influence decorin expression, which, in turn, promotes further calcification. The fact that overexpression of decorin did not induce calcification of BASMC in the absence of β-glycerophosphate points toward a role of decorin in enhancing vascular calcification in systems that are already committed to calcify.

In a previous study, we determined that the retroviral expression of bovine decorin in cultured endothelial cells yields a concentration of ∼1 μg decorin/mL culture medium over 24 hours. Moreover, in the present study, the addition of 0.05 and 0.5 μg/mL decorin increased calcification, as well. In vitro studies showing increased hydroxyapatite formation in collagen gels in the presence of decorin used concentrations of >1 μg/mL. The binding of decorin to hydroxyapatite was demonstrated at concentrations >1 mg/mL. In cell culture experiments, addressing intracellular signaling of decorin such as activation of the Akt/protein kinase B (Akt/PKB) pathway would be of interest. However, such studies have not been reported yet.
BASMC and that decorin overexpression and addition of purified decorin strongly increase calcification of BASMC. The concentrations of decorin used in the current study seem to be well in the range of concentrations that have been demonstrated to cause specific responses. Our findings that adding exogenous decorin or increasing decorin by overexpression causes a 3- to 4-fold increase in calcification argue for a role for this proteoglycan in promoting calcification.

Taken together, the present in vitro experiments demonstrate that decorin is strongly upregulated in calcifying cultures of BASMC and that decorin overexpression and addition of purified decorin strongly increase calcification of BASMC.

However, the mechanism mediating the calcification of BASMC in response to decorin is not known. Possible mechanisms are briefly discussed. Mineralization of bone requires an ECM framework containing collagen type 1. Similarly, in calcifying atherosclerotic plaques and in in vitro models of vascular calcification, mineralization occurs in association with collagen type 1. The ECM of bone also contains numerous noncollagenous matrix molecules such as proteoglycans, fibronectin, osteopontin, and osteonectin, which are thought to be important regulators of bone mineralization, because they modulate collagen fibril formation and orientation and, in addition, directly control apatite crystal growth. Studies from our laboratory have shown that retroviral overexpression of decorin induces collagen gel contraction and stimulates collagen synthesis, increasing fibronectin fibrillogenesis, and leads to the formation of a dense collagenous matrix in the intima of injured arteries in vivo. Therefore, it may be that decorin promotes the assembly of other ECM components such as collagen and fibronectin, which in turn serve as initiating or nucleating sites for hydroxyapatite crystal growth.

Furthermore, direct binding of decorin to hydroxyapatite might be involved in the effect of decorin on calcification. In cell-free assays, decorin and biglycan bind to hydroxyl apatite crystals but were shown to reduce hydroxyapatite crystal growth. At first, the findings obtained in cell-free assays seem to contradict the present results. However, the presence of other matrix molecules, growth factors, and phospholipid surfaces changes the effect of SLRPs dramatically. It has been shown that the inhibitory effect of SLRPs on hydroxyapatite crystal growth is reduced by collagen type I and that calcification of collagen gels is even enhanced by SLRPs. The latter finding supports our results in cultured SMC.

The association of decorin with calcium deposits in lesions from patients undergoing sudden death suggests that the current findings in cultured SMCs might be of relevance for calcification of human plaques. Interestingly, in human atherosclerosis, macrophages colocalize with both decorin and calcium deposits. Because macrophages release IL-1, which induces decorin expression in vascular SMC, it is conceivable that macrophages may cause increased calcification of atherosclerotic plaques by inducing decorin expression. Furthermore, the present findings might be of relevance during diabetes, because decorin has a glucose response element in its promoter. Accordingly, decorin is induced by high glucose concentrations in cultured mesangial cells and in mouse models of type 1 diabetes. Therefore, it seems possible that decorin plays a role in the severe vascular calcification occurring in diabetic patients.

**Acknowledgments**

Bovine decorin was kindly provided by Dr Kathryn Vogel (University of New Mexico, Albuquerque, NM), and LF-94, LF-136, PG28 cDNA, and P-16 cDNA were from Dr L. Fisher (Craniofacial and Skeletal Disease Branch, National Institutes of Dental Research, National Institutes of Health, Bethesda, Md). This work was supported by National Institutes of Health grants HL 18645 (T.N.W.) and HL62829 (C.M.G.), and by the Forschungskommission (Universitätsklinikum Düsseldorf, J.W.F.). Authors thank Ellen Briggs for assistance in preparation of the manuscript.

**References**


Decorin Promotes Aortic Smooth Muscle Cell Calcification and Colocalizes to Calcified Regions in Human Atherosclerotic Lesions

Jens W. Fischer, Susie A. Steitz, Pamela Y. Johnson, Allen Burke, Frank Kolodgie, Renu Virmani, Cecilia Giachelli and Thomas N. Wight

Arterioscler Thromb Vasc Biol. 2004;24:2391-2396; originally published online October 7, 2004;
doi: 10.1161/01.ATV.0000147029.63303.28

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/24/12/2391

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

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

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