Fibronectin and Collagen I Matrixes Promote Calcification of Vascular Cells in Vitro, Whereas Collagen IV Matrix Is Inhibitory

Karol E. Watson, Farhad Parhami, Victoria Shin, Linda L. Demer

Abstract—Vascular calcification is a frequent component of atherosclerosis, yet the pathological mechanisms that regulate its formation are poorly understood. Calcification of the vessel wall may represent a process by which cells that normally exhibit a smooth muscle phenotype differentiate into cells that exhibit an osteoblast-like phenotype. One of the determinants of cellular phenotype is extracellular matrix; thus, we undertook the current study to evaluate the influence of extracellular matrix on calcification of vascular cells in vitro. Cell lines derived from bovine aortic media were divided into 1 of 3 groups: those that did not mineralize, those that mineralized slowly, or those that mineralized rapidly. When slowly mineralizing cells were plated onto matrix produced by rapidly mineralizing cells, the time required for mineralization decreased from 33 ± 3.0 days to 7.8 ± 1.3 days. Matrix produced by rapidly mineralizing cells was found to contain 3 times the amount of collagen I and fibronectin but 70% less collagen IV than nonmineralizing clones. When slowly mineralizing cells were cultured on purified collagen I or fibronectin, mineralized nodule formation, calcium incorporation, von Kossa staining, and alkaline phosphatase activity increased. In contrast, culturing slowly mineralizing cells on purified collagen IV inhibited these mineralization parameters. Furthermore, blocking antibodies to α5 integrins significantly inhibited the fibronectin-mediated increases in alkaline phosphatase activity, indicating that integrin-based signaling may be involved. These data suggest that matrix composition can regulate development of arterial calcification and that a subpopulation of vascular cells preferentially produces positively regulating matrix components. (Arterioscler Thromb Vasc Biol. 1998;18:1964-1971.)

Key Words: atherosclerosis • calcification • extracellular matrix • osteogenesis

Extracellular matrix regulates many physiological and pathological processes. In the physiological process of bone development, specific extracellular matrix components are produced to create a unique environment that is permissive for the precipitation of calcium and phosphate ions as hydroxyapatite mineral. Similarly, in the pathological condition of atherosclerosis, characteristic extracellular matrix changes accompany development of the atherosclerotic lesion, and these changes significantly impact the course of the disease. A common feature of both bone and many atherosclerotic lesions is calcification. Vascular calcification is widespread in atherosclerosis, and in fact, this calcification shares many histological features with bone. This and other recent observations led us to hypothesize that development of arterial calcification may be regulated in a manner similar to development of bone. This hypothesis was strongly supported by the recent findings of Karsenty and associates: that endochondral ossification occurs in the aortas of matrix gα protein–knockout mice. We now hypothesize that the regulation is mediated, in part, through extracellular matrix signaling.

Vascular calcification may represent a process by which cells that normally exhibit a smooth muscle phenotype differentiate into cells that exhibit an osteoblast-like phenotype. Recently, we isolated such osteoblast-like cells from the artery wall and have termed them calcifying vascular cells (CVCs). CVCs were initially cloned from subpopulations of bovine aortic smooth muscle cells, and they exhibit several osteoblastic features in vitro—specifically, aggregation into nodules, spontaneous formation of hydroxyapatite mineral after noduleation, and production of bone differentiation factors. CVCs also produce extensive extracellular matrix that allows for in vitro assessment of matrix components. In the current study, we have used cloned CVCs to assess the role of extracellular matrix components on in vitro calcification. Our results reveal that 2 of the matrix components known to be important in bone formation, fibronectin and collagen, are also important in promoting mineralization of vascular cells, and that the matrix component collagen IV inhibits mineralization parameters. Furthermore, blocking antibodies to α5 integrins inhibit the fibronectin-mediated increases in alkaline phosphatase activity, indicating that integrin-based...
signaling may be involved. These data suggest that specific extracellular matrix molecules are capable of promoting arterial calcification and that a subpopulation of vascular cells preferentially produces these matrix components. These data also indicate a potential role for the α5 integrin and integrin-based signaling in the promotion of in vitro vascular calcification. Further research on the interplay between vascular cells, their extracellular matrix, and cellular integrins may lead to new therapeutic approaches to modify atherosclerotic calcification.

Methods

Cell Culture
Cloned bovine aortic medial cells were used for all in vitro studies. Nonmineralizing medial cell clones were bovine aortic smooth muscle cells (BASMCs), as identified by morphology, positive α-SM actin staining, and negative factor VIII-related antigen staining. Mineralizing medial cell clones were a subpopulation of BASMCs termed CVCs. CVCs were isolated from primary cultures of BASMCs termed CVCs, from these nodule-forming cultures, CVCs were cloned by limiting dilution and single-cell harvesting, and clonal lines were identified as CVCs by their positive staining with monoclonal antibody 3G5, as previously described, and by their ability to form calcified nodules in tissue culture. The standard tissue culture media for all cells was Dulbecco’s modified Eagle’s medium (Irvine Scientific) with 15% FBS (Hyclone Laboratories) supplemented with 1-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), penicillin (100 U/mL), and HEPES buffer adjusted to pH 7.25, all from Irvine Scientific. Cells were plated at a density of 10 000 cells/cm² with media changes every 72 hours.

Preparation of Cell-Synthesized Extracellular Matrix
Cell-synthesized extracellular matrix was prepared by allowing cells to grow on tissue culture plastic for 14 days followed by detachment of the cell monolayer with 25 mmol/L NH₄OH. For detachment, the monolayers were incubated with NH₄OH for 10 minutes, followed by extensive washing with calcium- and magnesium-free PBS (Gibco) and microscopic inspection of the dishes to ensure complete cell removal. If cells remained after the initial incubation, dilute alkaline treatment was repeated to complete cell removal. Cell-free extracellular matrices were maintained in tissue culture incubators with PBS for 2 weeks to ensure that no cells remained.

Indirect Immunofluorescent Staining
Immunofluorescent staining was performed on cultures of nonmineralizing, slowly mineralizing, and rapidly mineralizing cells. Cells were grown in chamber slides on tissue culture plastic for 24 hours before staining. The cultures were then fixed in 4% paraformaldehyde at room temperature for 5 minutes, followed by incubation with a blocking solution containing 5% BSA (vol/vol) and 3% nonimmune goat serum (vol/vol). Primary antibodies were applied for 2 hours, followed by “quenching” of autofluorescence with 0.2% NaHBF₄ for 15 minutes. FITC-conjugated secondary antibodies (Sigma Chemical Co) were then applied (1:30 dilution) for 30 minutes. After the slides were washed, mounting medium and a coverslip were applied, and the cells were examined by fluorescence microscopy (AxioScope 20; Carl Zeiss, Inc). Primary antibodies used were rabbit anti-foam collagen I, rabbit anti-foam collagen IV, rabbit anti-rat laminin, and rabbit anti-chicken fibronectin antibodies, all at a dilution of 1:50 (all antibodies obtained from Chemicon).

Immunoblotting
Cellular and extracellular matrix proteins were extracted from confluent monolayers of cells cultured for 14 days on tissue culture plastic. The proteins were extracted with lysis buffer containing 10 mmol/L HEPES, 200 mmol/L NaCl, 2 mmol/L CaCl₂, 1.5% Triton X-100, 1 mmol/L EDTA, 0.05% leupeptin, 0.07% pepstatin, and 0.2 mmol/L PMSF. The extracted proteins were separated on an 8% acrylamide gel (Novex X-Cell Mini-Cell) loaded with 12 μg protein per lane, followed by transfer onto 0.45-μm nitrocellulose membranes (Bio-Rad). Immunoblotting was performed with the ABC method (Vector Laboratories) and the following antibodies: rabbit anti-bovine collagen I, rabbit anti-bovine collagen IV, rabbit anti-rat laminin, and rabbit anti-chicken fibronectin (all antibodies obtained from Chemicon and diluted 1:100). Quantification of immunoblotting data was accomplished by calculating relative densitometric units for each band. This procedure consisted of multiplying the mean band density by the band area by using National Institutes of Health (NIH) Image software. For each blot, the band with the lowest densitometric unit was assigned a reference value of 1.0. Values obtained by densitometry were verified as being linearly related to the amount of protein on the blots by constructing standard curves for each matrix protein. Three separate immunoblots from 3 separate extractions were performed for each matrix molecule.

Experiments Performed on Purified Matrix Molecules
Slowly mineralizing cells were plated at a density of 10 000 cells/cm² onto purified collagen I, collagen IV, and fibronectin, or onto tissue culture plastic (all matrix molecules obtained from Sigma and coated at 10 μg/cm²) and cultured for 40 days. Cultures were observed for mineralized nodule formation and alkaline phosphatase activity. Quantification of mineralization was accomplished at the end of the 40-day culture period by multiplying the mean nodule density by the nodule area in each culture with the use of NIH Image software. Alkaline phosphatase determinations were performed every 4 days throughout the 40-day culture period. For alkaline phosphatase determinations, cells were lysed in buffer containing 0.2% NP40 in 1 mmol/L MgCl₂ at 4°C for 10 minutes. The cells were then scraped into an Eppendorf tube and sonicated for 10 seconds. Alkaline phosphatase activity was quantified by measuring release of p-nitrophenol from p-nitrophenyl phosphate (alkaline phosphatase assay; Sigma Diagnostics) and normalized to total cell number as determined by DNA content. Three separate experiments were performed.

45Ca Incorporation
Slowly mineralizing cells were plated at a density of 10 000 cells/cm² onto purified collagen I, collagen IV, and fibronectin, or onto tissue culture plastic. After 7 days, 1 μCi/mL of 45CaCl₂ was added and the cells cultured for an additional 48 hours. After this time, the medium was removed and the cells washed with PBS and scraped into scintillation vials. Phosphoric acid (0.2 mL) and 0.4 mL of 3% H₂O₂ were added to the vials, followed by incubation at 80°C for 60 minutes. After incubation, the mixture was dissolved in 0.6 mL of ethylene glycol monomethyl ether and counted in a scintillation counter.

Anti-Integrin Experiments
Anti-integrin α5 antibodies were purchased from Becton Dickinson and used at a concentration of 1 μg/mL. Slowly mineralizing cells were plated at a density of 10 000 cells/cm² onto either collagen I or fibronectin, and 24 hours later anti-integrin treatments began. Treatments were carried out every 3 days for 9 days total. Control antibodies were used as anti-CD3 antibodies (Dako Corporation) at an equivalent total protein concentration.

Statistics
All data are presented as mean±SD. Intergroup comparisons were performed by unpaired Student’s t test. Probability values of 0.05 or less were required for assumption of statistical significance.
Results

Classification of Vascular Cell Clones

In the current studies, we have classified subpopulations of bovine aortic medial cells into 3 types, based on their ability to form calcified nodules in tissue culture: (1) Nonmineralizing clones were defined as those that failed to form calcified nodules after >40 days of culture; (2) slowly mineralizing clones were defined as those requiring >28 days of culture before forming calcified nodules; and (3) rapidly mineralizing clones were defined as those forming calcified nodules within 10 days of culture.

Effect of Cell-Synthesized Extracellular Matrix on In Vitro Calcification (Matrix Swapping)

We evaluated the rate of mineralization of vascular cell clones when cultured on extracellular matrixes synthesized by different cells. Matrix synthesized by the 3 different types of clones was obtained as described above in Methods. Each clone was then plated either back onto its own matrix or onto matrix synthesized by 1 of the other cell types (matrix swapping).

(1) When nonmineralizing clones were plated onto any of the 3 matrix types, the cells remained nonmineralizing.

(2) When slowly mineralizing clones were plated onto either nonmineralizing matrix or onto slowly mineralizing matrix, they remained slowly mineralizing; however, when plated onto rapidly mineralizing matrix, such cells became rapidly mineralizing, with the time necessary for nodule formation to occur decreasing from 33±3.0 to 7.8±1.3 days (Table 1). Furthermore, by 21 days these nodules were heavily calcified, as demonstrated by von Kossa staining (Figure 1).

(3) When rapidly mineralizing clones were plated on any of the 3 matrix types, the cells remained rapidly mineralizing. Thus, a change in the rate of mineralization occurred only when slowly mineralizing cells were plated onto rapidly mineralizing matrix. These results suggest that mineralization can occur only when cells capable of mineralization are paired with a “permissive” extracellular matrix and that the rate of mineralization is determined, at least in part, by the composition of the matrix. A permissive matrix either can be supplied exogenously (as in the case of slowly mineralizing cells plated on rapidly mineralizing matrix) or can be pro-

TABLE 1. Time Required for Mineralization When Cells Were Plated Onto Different Cell-Synthesized Extracellular Matrixes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Matrix Type</th>
<th>N/A</th>
<th>6.3±1.0</th>
<th>7.5±1.0</th>
<th>7.6±1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmineralizing</td>
<td>Nonmineralizing</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Slowly mineralizing</td>
<td>Slowly mineralizing</td>
<td>32±3.2</td>
<td>33±3.0</td>
<td>7.8±1.3</td>
<td></td>
</tr>
<tr>
<td>Rapidly mineralizing</td>
<td>Rapidly mineralizing</td>
<td>8.6±1.0</td>
<td>7.5±1.0</td>
<td>7.6±1.6</td>
<td></td>
</tr>
</tbody>
</table>

Values represent days required for nodule formation to occur under each culture condition. Values are the mean ± SD of 8 separate wells per condition. N/A indicates that no nodule formation occurred under these culture conditions.

*P<0.05 for cells grown on rapidly mineralizing matrix vs cells grown on slowly mineralizing or nonmineralizing matrix.

Analysis of Matrix Molecules Produced

The composition and amount of matrix produced by each class of medial cell clone were next determined by immunofluorescent staining and immunoblotting. The molecules analyzed by immunofluorescent staining were laminin, collagen I, fibronectin, and collagen IV. Laminin was nearly undetectable in rapidly mineralizing clones (Figure 2C), as well as in slowly mineralizing and nonmineralizing clones (data not shown); thus, this molecule was not studied further. Collagen I, fibronectin, and collagen IV were all present in detectable amounts in rapidly mineralizing clones (Figure 2A, 2B, and 2D), as well as in slowly mineralizing and nonmineralizing clones (data not shown). The greatest amounts of collagen I and fibronectin were present in rapidly mineralizing clones, as determined by immunoblotting (Figure 3). Rapidly mineralizing clones produced 3 times more collagen than did either nonmineralizing or slowly mineralizing clones (Table 2). Rapidly mineralizing clones also produced 3 times more fibronectin than did slowly mineralizing clones and 16 times more fibronectin than did nonmineralizing clones (Table 2). Collagen IV revealed an opposite pattern of abundance, with nonmineralizing clones producing the highest amounts (Table 2). Nonmineralizing clones produced 70% more collagen IV as did rapidly mineralizing clones.

Effects of Purified Extracellular Matrix Molecules on Mineralized Nodule Formation, Calcium Incorporation, and Alkaline Phosphatase Activity

To further investigate the role of matrix on in vitro calcification, slowly mineralizing clones were plated onto the purified matrix molecules collagen I, fibronectin, and collagen IV to determine whether purified matrix molecules could reproduce the results obtained with the cell-synthesized extracellular matrix. Slowly mineralizing clones were plated onto the various matrices, cultured for a total of 40 days, and assessed for mineralized nodule formation and calcium in-
corporation, as well as alkaline phosphatase activity as a marker of osteoblastic differentiation.

Mineralized nodule formation was enhanced by growth on either collagen I or fibronectin and inhibited by growth on collagen IV, compared with tissue culture plastic. Cells grown on tissue culture plastic formed small, discrete nodules during the culture period as previously described24 (Figure 4A). When these cells were grown on either collagen I or fibronectin, much larger nodules were formed that appeared to be connected by large ridges of cells and extracellular material (Figure 4B and 4C). In contrast, growth on collagen IV significantly inhibited mineralized nodule formation (Figure 4D). When the area of mineralization was calculated in each culture, cells grown on collagen I or fibronectin produced 3.1 ± 1.1 times as much mineralization as did cells grown on tissue culture plastic, whereas cells grown on collagen IV produced only 0.21 ± 0.18 times the mineralization as cells grown on tissue culture plastic (Figure 5A).

Radiolabeled calcium incorporation also increased significantly. Cells grown on collagen I or fibronectin incorporated 2.2 ± 0.5 times the calcium as did cells grown on plastic, and cells grown on collagen IV incorporated only 0.05 ± 0.01

TABLE 2. Relative Amounts of Matrix Proteins Produced by Medial Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Collagen I</th>
<th>Fibronectin</th>
<th>Collagen IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapidly mineralizing</td>
<td>3.1 ± 1.0*</td>
<td>16 ± 3.8†</td>
<td>1.0 ± 0.0†</td>
</tr>
<tr>
<td>Slowly mineralizing</td>
<td>1.2 ± 0.22</td>
<td>5.3 ± 3.0</td>
<td>1.2 ± 0.05‡</td>
</tr>
<tr>
<td>Nonmineralizing</td>
<td>1.1 ± 0.6</td>
<td>1.0 ± 0.0</td>
<td>1.7 ± 0.14</td>
</tr>
</tbody>
</table>

Data presented are relative densitometric units derived by multiplying the mean immunoblot band density by the mean band area. For each blot, the band with the lowest densitometric unit was assigned a reference value of 1.0. Each value listed is the mean of 3 independent immunoblotting experiments.

*P < 0.05 for rapidly mineralizing vs slowly mineralizing cells.
†P < 0.05 for rapidly mineralizing vs nonmineralizing cells.
‡P < 0.05 for slowly mineralizing vs nonmineralizing cells.
times the calcium as did cells grown on plastic (Figure 5B). All calcium incorporation experiments were performed at confluence. At confluence, there was no difference in cell number, regardless of the matrix on which the cells were plated.

Alkaline phosphatase activity was also enhanced by growth on either collagen I or fibronectin. Slowly mineralizing clones grown on collagen I or fibronectin expressed up to 8 times greater alkaline phosphatase activity compared with clones grown on either collagen IV or tissue culture plastic.

**Figure 4.** Morphological changes with long-term culture on purified matrix molecules. Slowly mineralizing cells were plated at a density of 10,000 cells/cm² and cultured for 40 days on either tissue culture plastic (A), collagen I (B), fibronectin (C), or collagen IV (D). Cells grown on tissue culture plastic formed small, discrete nodules during the culture period (single arrowheads). Larger mineralized nodules connected by ridges of matrix-rich material were formed on collagen I and fibronectin (multiple arrowheads). Nodule formation was nearly absent on collagen IV. Bar, 100 μm.

**Figure 5.** Changes in mineralized nodule formation (A) and radiolabeled calcium incorporation (B) with long-term culture of slowly mineralizing cells on purified matrix molecules. Mineralized nodules were manually outlined on digitized images, and the enclosed area was multiplied by the nodule density to derive relative mineralization units. Mineralization, defined in this manner, was significantly increased when cells were grown on either collagen I or fibronectin and decreased when cells were grown on collagen IV. Values represent mean±SD of area averaged over 3 experiments. *P<0.05 compared with cells grown on plastic. Calcium incorporation, measured as incorporated radioactivity after a 48-hour incubation with ⁴⁵Ca, was significantly increased when cells were grown on either collagen I or fibronectin and decreased when cells were grown on collagen IV. All calcium incorporation experiments were performed at confluence. At confluence, there was no difference in cell number, regardless of the matrix on which the cells had been plated. Values represent the mean±SD of quadruplicate wells from a representative of 2 experiments. *P<0.05 compared with cells grown on plastic.
This increase began at 6 days of culture (Figure 6A), and this relationship held even when alkaline phosphatase activity was controlled for cell number (Figure 6B). When rapidly mineralizing cells were grown on the various matrix molecules, there was a high level of nodule formation, alkaline phosphatase activity, and calcium incorporation, and none of these parameters was altered by growth on specific matrix molecules, with the exception that calcium incorporation decreased on collagen IV (data not shown). This finding again is consistent with the hypothesis that mineralization occurs when cells capable of mineralization are paired with a permissive extracellular matrix. The permissive matrix can either be supplied exogenously or produced by the cells themselves (rapidly mineralizing clones).

Anti-Integrin Treatment of Slowly Mineralizing Cells

Treatment of slowly mineralizing clones with blocking antibodies to α5 integrins resulted in a decrease in alkaline phosphatase activity over a 7-day treatment period. For cells grown on fibronectin, which is an α5 ligand, the decrease was 77 ± 33% and statistically significant (P < 0.013) (Figure 7). For cells grown on collagen I, which is not an α5 ligand, the decrease was 34 ± 14% and not statistically significant. This decrease in alkaline phosphatase activity when cells grown on fibronectin were treated with blocking antibodies to the α5 integrin suggests that integrin-based signaling is involved in the matrix modulation of alkaline phosphatase activity.

Figure 6. Changes in alkaline phosphatase activity with long-term culture on purified matrix molecules. Alkaline phosphatase activity was significantly greater in cells grown on collagen I and fibronectin. A, Alkaline phosphatase activity per well. B, Alkaline phosphatase activity normalized to cell number, determined by DNA content. Values represent the mean ± SD of quadruplicate wells from a representative of 3 separate experiments.

Figure 7. Effect of anti-integrin α5 antibodies on alkaline phosphatase activity in slowly mineralizing clones grown on collagen I or fibronectin. An equal amount (1 μg/mL) of either blocking antibody to the α5 integrin or antibody to CD3 (control) was added to cultures every 3 days for a total of 9 days. When cells grown on collagen I were treated with antibodies to α5 integrins, there was a slight, but nonsignificant, decrease in alkaline phosphatase activity, compared with cells grown on collagen I treated with control antibody. When cells grown on fibronectin were treated with antibodies to α5 integrins, however, there was a statistically significant (P = 0.013) decrease in alkaline phosphatase activity, indicating that integrin-based signaling may be involved. Values represent mean ± SD of quadruplicate wells from a representative of 3 separate experiments.
Discussion
Most cells secrete a complex extracellular matrix that is crucial for maintenance of normal cellular function. In addition to mechanical and structural support, extracellular matrix controls many aspects of cell behavior, such as attachment, migration, differentiation, and gene expression. Extracellular matrix is of paramount importance in the formation of calcified structures, as evidenced by experiments showing that a matrix of appropriate composition and organization, even in the absence of cells, can become calcified. Thus, it appears that physiological calcification is based on the ability of specialized cells to secrete a complex extracellular matrix that is permissive for mineralization. Mineralization is usually confined to specific locations in vivo, and only specialized cells are able to secrete a permissive matrix. When calcification is uncontrolled, it may contribute to the pathogenesis of a variety of diseases, including atherosclerosis. Ectopic calcification is a prominent feature of atherosclerosis, and until recently, the cells responsible for this mineralization were not known. We have recently discovered a subpopulation of cells isolated from the media of aortas that have osteoblast-like properties and that in vitro are capable of forming a calcified matrix. In the current studies, we have begun to characterize the regulatory role of the extracellular matrix produced by these vascular cells that calcify, and our results indicate that CVCs secrete a complex extracellular matrix that can influence the rate and amount of calcification produced in vitro. The predominant matrix components produced by mineralizing cells, collagen I and fibronectin, were found to promote calcification, whereas collagen IV was inhibitory.

Collagen I and fibronectin are known to be critical in the formation of calcified structures and are associated with osteoblast differentiation. Collagen I comprises over 90% of the organic compartment of mature bone, and type I collagen fibrils are believed to be the "scaffolding" necessary for mineralization. During embryogenesis, formation of the core of limb buds coincides with a transient expression of collagen I and fibronectin. As mesenchymal precursors differentiate into osteoblasts, collagen I, fibronectin, and alkaline phosphatase are upregulated. In addition, fibronectin is present in the condensing core of chick limb bud mesenchyme during cartilage and bone differentiation but is completely absent from the adjacent muscle.

The role of extracellular matrix in atherosclerosis is increasingly recognized and atherosclerotic calcification represents an extreme case of matrix alteration. Vascular cells are known to synthesize their own complex extracellular matrix, and this synthesis is affected by the pre-existing matrix as well as the state of differentiation of the cell. There are 2 major classes of matrix in the vessel wall: the basal lamina and the interstitial matrix. The basal lamina includes collagen IV, laminin, and heparan sulfate proteoglycans, whereas the interstitial matrices include collagen I, collagen III, fibronectin, elastin, and chondroitin sulfate proteoglycans. Numerous changes in the extracellular matrix occur in atherosclerosis, and alterations in the composition of artery wall matrix contributes to atherogenesis. Just as in the development of calcified structures, collagen I and fibronectin have also been specifically implicated in the development of atherosclerotic lesions. Intimal sclerosis and lesion progression involve increased expression of collagen I, and investigators have also demonstrated that phenotypic modulation of vascular cells occurs when the cells are grown on collagen I or fibronectin but that this modulation is inhibited when cells are grown on elastin or laminin.

In addition to direct effects of matrix molecules on the cells, matrix-bound growth factors may also modulate calcification. Extracellular matrix specifically binds cytokines and growth factors, affecting both their availability and biological activity. One growth factor that may be particularly important in vascular calcification is transforming growth factor-β1 (TGF-β1). TGF-β1 is bound by a variety of matrix molecules, including fibronectin, and expression of the TGFβ-1 gene is regulated by extracellular matrix. TGF-β1 is thought to be a major contributor to the sclerosis and fibrosis seen in atherosclerosis, as well as the end-stage fibrosis of parenchymal organs such as the liver, lungs, and kidneys. It has been proposed that many of the biological effects of TGFβ-1 are mediated by its ability to regulate the synthesis of extracellular matrix, and TGF-β1 is known to greatly increase the production of collagen I and fibronectin.

We have previously shown TGF-β1 to greatly increase the formation of calcified nodules by CVCs. The current results suggest that the mechanism of this effect may be through alterations in matrix synthesis or composition.

Integrins are a large family of heterodimeric transmembrane receptors that mediate attachment of cells to the extracellular matrix. The integrin receptor family is composed of distinct α and β subunits that heterodimerize to form receptors with characteristic binding specificities. As receptors for the extracellular matrix, integrins mediate a variety of signals that regulate several important events, including differentiation. The α5 integrin subunit heterodimerizes with the β1 subunit to form a receptor for fibronectin. In the current studies, blocking antibodies to the α5 subunit inhibited the fibronectin-mediated increase in alkaline phosphatase by CVCs. When CVCs were plated on a collagen I substrate, there was a slight (nonsignificant) decrease in alkaline phosphatase, and because cells plated on collagen I may continue to produce their own fibronectin, some decrease in alkaline phosphatase activity might be expected. The decrease seen in alkaline phosphatase activity when CVCs plated on fibronectin were treated with the blocking antibody to the α5 integrin was much greater and suggests that integrin-based signaling is involved. Because the anti-α5 antibody has not previously been shown to be neutralizing in bovine cells, however, the possibility of a specific effect of this antibody unrelated to integrins cannot be excluded.

Several diseases as well as normal physiological processes are known to be modulated by changes in the extracellular matrix. The current results reveal that extracellular matrix changes also modulate in vitro vascular calcification. Further research on the interplay between vascular cells, their extracellular matrix, and cellular integrins may lead to new therapeutic approaches to treat atherosclerotic calcification.
Acknowledgments
This work was supported in part by NIH grants HL-30568 and HL-07412, the Stresand Research Fund established by the Lincy Foundation, and the Laubisch Fund (all awarded to L.L.D.). Dr Watson is a Merck Fellow of the American College of Cardiology. The authors are grateful to James Howard, Denise Park, and Amethyst Vu for technical assistance and to Dr Joy Frank for suggestions.

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doi: 10.1161/01.ATV.18.12.1964

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

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