Calcification of Advanced Atherosclerotic Lesions in the Innominate Arteries of ApoE-Deficient Mice
Potential Role of Chondrocyte-Like Cells

Marcello Rattazzi, Brian J. Bennett, Florian Bea, Elizabeth A. Kirk, Jerry L. Ricks, Mei Speer, Stephen M. Schwartz, Cecilia M. Giachelli, Michael E. Rosenfeld

Objective—Advanced atherosclerotic lesions in the innominate arteries of chow-fed apolipoprotein E–deficient mice become highly calcified with 100% frequency by 75 weeks of age. The time course, cell types, and mechanism(s) associated with calcification were investigated.

Methods and Results—The deposition of hydroxyapatite is preceded by the formation of fibro-fatty nodules that are populated by cells that morphologically resemble chondrocytes. These cells are spatially associated with small deposits of hydroxyapatite in animals between 45 and 60 weeks of age. Immunocytochemical analyses with antibodies recognizing known chondrocyte proteins show that these cells express the same proteins as chondrocytes within developing bone. Histological and electron microscopic analyses of lesions from animals between 45 and 60 weeks of age show that the chondrocyte-like cells are surrounded by dense connective tissue that stains positive for type II collagen. Nanocrystals of hydroxyapatite can be seen within matrix vesicles derived from the chondrocyte-like cells. In mice between 75 and 104 weeks of age, the lesions have significantly reduced cellularity and contain large calcium deposits. The few remaining chondrocyte-like cells are located adjacent to or within the large areas of calcification.

Conclusions—Calcification of advanced lesions in chow-fed apolipoprotein E–deficient mice occurs reproducibly in mice between 45 and 75 weeks of age. The deposition of hydroxyapatite is mediated by chondrocytes, which suggests that the mechanism of calcification may in part recapitulate the process of endochondral bone formation.

Key Words: atherosclerosis ■ calcification ■ chondrocytes ■ apolipoprotein E–deficient mice

Many advanced human atherosclerotic lesions contain deposits of calcium phosphate. The significance of calcium deposition with regard to clinical events is still a matter of debate. Currently, the mechanisms by which advanced lesions become calcified are unknown, and may include both dystrophic calcification as well as direct ossification. The presence of ossified bone within plaques and the expression of osteogenic cell makers has been previously reported. The cell-mediated process of bone formation involves both osteoblasts and chondrocytes. Recent evidence suggests that both of these cell types may play a similar role in mediating the calcification of atherosclerotic lesions in humans and mice. Chondrocyte metaplasia within blood vessels has been previously reported in humans and is frequently observed in mouse models of atherosclerosis. The designation of these cells as chondrocytes is based on the morphological similarity to chondrocytes in cartilage and on the presence of cartilage and bone extracellular matrix proteins.

This study is a continuation of our characterization of advanced atherosclerotic lesions in the innominate arteries of chow-fed apolipoprotein E–deficient (apoE–/–) mice. We previously reported that unstable lesions in the innominate arteries initially contain large central necrotic cores covered by thin fibrous caps, and that these plaques are converted to more stable fibro-fatty nodules by 1 year of age. This conversion is associated with the presence of chondrocyte-like cells. In the current study, we have evaluated the time course of calcification and the temporal and spatial association between the chondrocyte-like cells and the deposition of hydroxyapatite. In addition, we have used immunocytochemistry and electron microscopy to further define both the phenotype of the chondrocyte-like cells and the mechanism of calcification. The data suggest that calcification is initiated in younger mice and becomes apparent in mice between 45
and 60 weeks of age, and that the chondrocyte-like cells are responsible for depositing hydroxyapatite through a process that may recapitulate the cellular and temporal aspects of endochondral ossification.

Methods

Animals

Male and female apoE−/− mice on a C57BL/6J background (n=50) were fed normal chow and water ad libitum throughout the study. Forty mice were euthanized by lethal injection (Ketamine/Xylaject; 28 between 45 and 75 weeks of age, and 12 between 75 and 104 weeks of age) and perfused with PBS at physiological pressure followed by 10% buffered formalin through the left ventricle. An additional subset of mice (n=4) was perfused only with PBS for frozen tissue analysis. Tissues from 6 mice were used for electron microscopic analysis. The femoral and humeral bones from two newborn mice were also included in this study. All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Preparation and Analysis of Tissue

The base of the right carotid artery (also called the brachiocephalic trunk or the innominate artery) was dissected out, embedded in paraffin, and serially sectioned (5 μm). Every twenty-fifth section was stained with a modified Movat pentachrome stain.17 To identify vascular calcification, adjacent sections were stained with the von Kossa and Movat stains to identify calcified areas and matrix vesicles.

Immunochemistry

Sections adjacent to those stained with the Movat and von Kossa stains were stained with an anti-mouse macrophage antibody (Mac-2; Accurate Chemical), anti-smooth muscle actin antibody (1A4; Dako), type II collagen antibody (Novocastra), anti-osteoprotegerin (OPG; Santa Cruz Biotechnology), anti-receptor activator of NF-κB ligand (RANKL; Santa Cruz Biotechnology), anti-parathyroid hormone related peptide (PTHRP; Santa Cruz Biotechnology), and anti-osteopontin (OPN; R&D Systems). Collagen II staining was done after antigen retrieval with trypsin (Zymed). Control sections were incubated with rat, goat, or rabbit IgG (Zymed). The number of chondrocyte-like cells that stained positively for OPG, PTHrP, RANKL, and OPN was established in 28 animals between 45 and 75 weeks of age and expressed as a percentage of the total number of chondrocyte-like cells that were counted in the adjacent Movat stained section.

Alkaline Phosphatase Activity

Cryosections of the innominate artery (8 μm) from 4 mice between 45 and 60 weeks of age were directly incubated with the working solution used for detecting alkaline phosphatase activity (ALP) conjugated antibodies (Red Alkaline Phosphatase Substrate Kit I, Vector Labs, Burlingame, CA). Adjacent sections were stained with the von Kossa and Movat stains to identify calcified areas and cellular composition, respectively.

Statistical Analysis

All data were expressed as mean±SD. Significant differences between means were determined by the nonparametric Mann-Whitney U test (STATA, Intercooled version 8).

Results

Histology

Fibro-fatty nodules in the advanced lesions in the innominate arteries are a nidus for calcification and become highly calcified in mice at 75 weeks of age. However, the process of calcification is initiated in much younger mice and becomes apparent in mice between 45 and 60 weeks of age where small deposits of hydroxyapatite are observed (Figure 1A). These small deposits range from 17% to 34% of the plaque area (Table 1). The lesions exhibit a high degree of cellularity, ranging between 800 and 1500 cells per mm² of lesion area, with an average of ~13% of the cells being chondrocyte-like cells (Table 1). The chondrocyte-like cells are located both adjacent to and within areas of calcification, as well as in areas devoid of calcium (Figure 1A; Figures I through III, available online at http://atvb.ahajournals.org). Many of the chondrocyte-like cells are surrounded by a dense
ring of connective tissue analogous to the appearance of chondrocytes within growth cartilage (Figure 3A). This ring of connective tissue stains positively with an antibody recognizing type II collagen (Figure 1B).

In mice between 75 and 104 weeks of age, there is a 100% frequency of calcification and the deposits are much larger, reaching an average of ≈50% of lesion area (Figure 2 and Table 1). These extremely advanced plaques also contain significant amounts of connective tissue and cholesterol clefts but have very few remaining cells (Table 1). The remaining chondrocyte-like cells have condensed pycnotic nuclei and are situated adjacent to or within the areas of calcium deposition (Figure 2D). Starting at 60 weeks of age, there is also a high frequency of chondrocyte-like cells and calcification in the medial layer (observed in ≈60% of the lesions; Figure IV, available online at http://atvb.ahajournals.org). This medial calcification is often associated with breaks in the internal elastic lamina (Figure 2C) and is observed in >80% of the lesions by 75 weeks of age.

**Electron Microscopy**

Electron microscopic analysis of the lesions from mice between 60 and 75 weeks of age revealed that the extracellular matrix between chondrocyte-like cells is rich in collagen fibrils and vesicular material. Many of the cells are associated with early stages of calcium deposition (Figure 1C through 1F), where the matrix surrounding these cells contains needle-like structures compatible with nanocrystals of hydroxyapatite (Figure 1D through 1F). There are also vesicular structures which, based on their shape and size, resemble matrix vesicles. Some of these vesicles contain electron dense needle-like structures that appear to be associated with the initial process of mineralization (Figure 1F).

**Immunocytochemical Analyses**

Immunocytochemistry with antibodies specific for chondrocyte proteins was used to further characterize the phenotype of the chondrocyte-like cells. In addition, as a direct basis of comparison we generated sections of the growth plates of bones obtained from newborn apoE−/− mice. The Movat pentachrome staining of these sections of bone showed the characteristic zones containing proliferative and hypertrophic chondrocytes (Figure 3A). Staining patterns in the bone were compared with the expression patterns by chondrocyte-like cells within the advanced atherosclerotic lesions of mice between 45 and 60 weeks of age (Figures 3 and 4 and Table 2). As shown in Figure 3, resting/proliferative chondrocytes in the developing bone stain with antibodies for OPG and PTHrP (Figure 3C and 3D) and are surrounded by a matrix rich in type II collagen (Figure 4B). The resting/proliferative chondrocytes (that are not actively involved in calcification) do not stain for OPN and sporadically stain with anti-RANKL (Figure 4E and 4F). In contrast, hypertrophic chondrocytes that are actively engaged in the ossification process express all of the markers.

As shown in Figure 4, chondrocyte-like cells in the lesions do not stain with antibodies recognizing α-actin or Mac-2. The staining for α-actin is restricted to the medial layer and the fibrous cap of the lesions (Figures 4C and 4B). Mac-2 predominately stains lateral xanthomas, aggregates of lipid loaded macrophage-derived foam cells (Figures 4B and 4C). In contrast, in noncalcified lesions, only chondrocyte-like cells stain with antibodies specific for OPN, RANKL, and PTHrP (Figures 4D through 4F and 4H through 4I). Very few chondrocyte-like cells express OPN (<20% of the chondrocyte-like cells) (Figures 4G, 4I, and 4E; Table 2). OPN staining colocalizes primarily with the cells that are positive for Mac-2 (Figures 4B and 4C). In both calcified and noncalcified plaques, ≈80% of the chondrocyte-like cells stain positive for OPN, 70% for PTHrP, and ≈50% of them

**Table 1. Temporal Changes of Calcified Plaques**

<table>
<thead>
<tr>
<th>Weeks of Age</th>
<th>45 (n=10)</th>
<th>60 (n=10)</th>
<th>75 (n=10)</th>
<th>104 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cellularity, cells per mm² of lesion area</td>
<td>1529.9±674.1</td>
<td>824.7±328.8</td>
<td>462.3±206.5</td>
<td>200.9±102.2</td>
</tr>
<tr>
<td>Chondrocyte-like cells, cells per mm² of lesion area</td>
<td>211.1±137.7 (13.8%)</td>
<td>109.6±67.6 (13.3%)</td>
<td>28.7±10.6 (6.2%)</td>
<td>19.3±14.4 (9.6%)</td>
</tr>
<tr>
<td>Calcified area, % of total lesion area</td>
<td>17.6±15.9</td>
<td>33.9±18.1</td>
<td>48.9±19.4</td>
<td>51±17.5</td>
</tr>
</tbody>
</table>

Values shown are means±SD, value in parentheses is the percentage of total cells.

**Figure 2.** Calcification of advanced atherosclerotic lesions in the innominate arteries of 2-year-old apoE−/− mice. Diffuse calcification occupies the majority of the lesion (A) or can occur within a smaller fibro-fatty nodule (B, N indicates nodule). Calcification often disrupts the internal elastic lamina (C, arrow points to disrupted internal elastic lamina). Chondrocyte-like cells within large calcified areas have condensed pycnotic nuclei (D, arrows point to condensed nuclei). A and B, 100× magnification; C and D, 400×. Movat pentachrome stain.
express RANKL (Figures 4C, 4D, 4F, II, and III; Table 2). In calcified plaques there is increased staining for OPN by chondrocyte-like cells (>30% of cells, Figure III). Medial chondrocyte-like cells do not express α-actin or mac-2 but are von Kossa and ALP positive (Figure 5) and surrounded by type II collagen (Figure IV).

**Alkaline Phosphatase Activity**

Alkaline phosphatase is a key enzyme expressed by osteogenic cells during bone formation. As expected, there is ALP activity within the growth plate of the bones adjacent to the calcification zone (data not shown). There is also ALP activity in chondrocyte-like cells situated predominantly in areas surrounding the zone of mineralization in the advanced atherosclerotic lesions as demonstrated by Von Kossa staining (Figure 5). Areas without chondrocyte-like cells or not surrounding mineralization are devoid of ALP activity.

**Discussion**

This study is a continuation of our characterization of advanced atherosclerotic lesions in the innominate arteries of chow-fed apoE−/− mice and focuses on plaque calcification, a process that also occurs with high frequency in older humans. We observed that small areas of calcification are apparent in many of the mice between 45 and 60 weeks of age and that by 75 weeks of age there is a 100% frequency of calcification. Furthermore, it appears that the chondrocyte-like cells are responsible for depositing the hydroxyapatite through a process that may recapitulate the cellular and temporal aspects of endochondral ossification. This conclusion is supported by the temporal and spatial association between the chondrocyte-like cells and hydroxyapatite deposits, the concordance in the patterns of expression of the chondrocyte markers by cells within both the developing bone and the advanced atherosclerotic lesions (Table 2), the presence of active alkaline phosphatase in the chondrocyte-like cells within and adjacent to areas of calcification, and by electron microscopic data showing matrix vesicles derived from the chondrocyte-like cells containing electron dense crystalline material consistent with hydroxyapatite.

The detection of OPG and RANKL in the mouse plaques is consistent with previous reports of RANKL and OPG expression in human plaques. It is currently unclear what role these molecules play within the plaques. However, recent epidemiological studies show that serum OPG levels are associated with the extent of atherosclerosis and calcification in humans and may predict the progression of atherosclerosis and cardiovascular mortality. The OPG−/− mouse is characterized by an osteoporotic phenotype and development of extensive medial calcification of the major blood vessels. To determine whether OPG regulates intimal calcification and atherosclerosis progression in the apoE−/− mouse lesions, we are currently generating OPG−/−×apoE−/− double knockout mice.

Osteopontin is normally expressed in bone and plays a role in regulating the process of mineralization. OPN is expressed by smooth muscle cells, endothelial cells, and macrophages in human atherosclerotic lesions especially in calcified areas. In the atherosclerotic lesions of the apoE−/− mice the chondrocyte-like cells show a similar pattern of OPN expression, staining positive for OPN more so when associated with areas of calcification. Both in vivo and vitro studies suggest that OPN may act locally to inhibit calcification by binding hydroxyapatite and reducing crystal growth. PTHrP is a critical mediator of chondrocyte growth and maturation as mice deficient in PTHrP have severe abnormalities in bone development. PTHrP is present in human atherosclerotic lesions, but whether it plays a role in the atherogenic process has not been determined.

ALP is crucial for initiating mineralization in bone. ALP activity has been shown inside matrix vesicles shed from chondrocytes and most likely works to increase the availabil-
ity of inorganic phosphate (Pi) needed for hydroxyapatite crystal growth.\(^3\) Increases in ALP activity is a marker of the transition to an osteogenic phenotype by smooth muscle cells in vitro. ALP activity is increased after exposure of smooth muscle cells to inflammatory factors.\(^3\)–\(^3\) In the present studies, ALP activity was documented only in areas of active calcification and in areas rich in chondrocyte-like cells and suggests that increases in ALP activity plays an active role in the deposition of hydroxyapatite within the apoE\(^+/-\) mouse lesions.

Matrix vesicles are believed to play a crucial role in the formation of the seed crystals of hydroxyapatite within the lumen of the vesicles.\(^3\) The presence of matrix vesicles associated with the chondrocyte-like cells in the lesions of the apoE\(^+/-\) mouse (Figure 1) is consistent with previous observations in calcified human lesions.\(^3\) However, it is also possible that these vesicles are apoptotic bodies rather than matrix vesicles, because apoptotic bodies have also been implicated in vascular calcification.\(^3\)

The source of the osteogenic cells in the advanced lesions of the apoE\(^+/-\) mouse is unknown. However, in vitro studies have shown that vascular smooth muscle cells can undergo a phenotypic switch characterized by the loss of expression of smooth muscle cell markers and the gain of expression of molecules characteristic of osteoblasts and chondrocytes.\(^3\) In the advanced lesions of the apoE\(^+/-\) mouse, the staining of \(\alpha\)-actin was limited to the media or fibrous cap regions and was not expressed by the chondrocyte-like cells. This is consistent with the possibility that smooth muscle cells within the intima are converted to chondrocyte-like cells in response to signals such as elevated levels of Pi, or factors such as tumor necrosis factor-\(\alpha\), interleukin-6, and transforming growth factor-\(\beta\), which can modulate the smooth muscle cell phenotype.\(^3\)–\(^3\),\(^3\) However, it is also feasible that mesenchymal or hematopoietic stem cells are recruited into the plaques and are the source of the chondrocytes. For example, calcifying vascular cells (CVCs) retain a multi-lineage potential that is analogous to mesenchymal stem cells because these cells can be induced to express markers of chondrocytes, smooth muscle cells, and marrow stromal cells.\(^3\)

Like humans, the arteries of older apoE\(^+/-\) mice become calcified. The reproducibility of calcification in the lesions in the innominate arteries of the older apoE\(^+/-\) mouse now provides a model in which the mechanisms that mediate plaque calcification can be investigated and in which interventions that target plaque calcification can be tested. Fur-

### TABLE 2. Comparison of the Expression Pattern of Chondrocyte Markers by Chondrocyte-Like Cells in Advanced Atherosclerotic Lesions With Chondrocytes in Developing Bone

<table>
<thead>
<tr>
<th>Markers</th>
<th>Artery Chondrocyte-Like Cells in Noncalcified Areas</th>
<th>Artery Chondrocyte-Like Cells in Calcified Area</th>
<th>Bone Resting/Proliferative Chondrocytes in Bone</th>
<th>Bone Hypertrophic Chondrocytes in Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM actin</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mac-2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OPG</td>
<td>80%±18%</td>
<td>80%±13%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PTHrP</td>
<td>70%±15%</td>
<td>65%±19%</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>RANKL</td>
<td>52%±20%</td>
<td>52%±12%</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OPN</td>
<td>19%±8%</td>
<td>34%±8%*</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Collagen II</td>
<td>100%</td>
<td>100%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Von Kossa</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>ALP activity</td>
<td>+/-</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

NA indicates not applied. Percentages reflect the percentage of all cells with chondrocyte-like morphology in the Movat stained sections that stained positively with antibodies to the listed proteins in adjacent sections. + indicates majority of cells stained positive; −, absence of staining; +/-, very small numbers of cells stained positive for the respective markers. Serial sections from 28 animals between 45 and 75 weeks of age were included in the immunocytochemical studies with 14 lesions that were calcified and 14 without calcification as assessed by von Kossa staining of adjacent sections. ALP activity was assessed in separate frozen sections from 4 animals between 45 and 75 weeks of age. Sections of bone were derived from 2 newborn apoE\(^-/-\) mice.

\(^*\)P≤0.002 with respect to the percentage of cells in the noncalcified areas.

Figure 5. Chondrocyte-like cells within areas of calcification in advanced atherosclerotic lesions of older apoE\(^-/-\) mice contain active alkaline phosphatase. This figure shows serial sections of advanced lesions in the innominate arteries from 3 different mice between 60 and 75 weeks of age. A, D, and G, Movat pentachrome stain; D, E, and H, von Kossa stain; C, F, and I, alkaline phosphatase activity. A through F, 400× magnification; G through I, 100× magnification.
thermore, coupled with studies on the modulation of cellular phenotypes and the role of stem cells, this model may enable us to determine the source of osteogenic cells and to elucidate the factors and signals that recruit or induce differentiation of osteogenic cells within the setting of atherosclerosis.

Acknowledgments

These studies were supported by National Institutes of Health grants HL-01014 (to C.M.G.), HL-72262 (to S.M.S.), and HL-076748 (to M.E.R.) and funding provided by the Fondazione Cassamarca di Treviso (to M.R.).

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Arterioscler Thromb Vasc Biol. 2005;25:1420-1425; originally published online April 21, 2005; doi: 10.1161/01.ATV.0000166600.58468.1b
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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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