Acetylated Low-Density Lipoprotein Stimulates Human Vascular Smooth Muscle Cell Calcification by Promoting Osteoblastic Differentiation and Inhibiting Phagocytosis

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Background—Vascular smooth muscle cells (VSMCs) in atherosclerotic lesions display an osteogenic phenotype, and calcification commonly occurs in association with lipid. We therefore tested the hypothesis that lipid components in atherosclerotic lesions influenced VSMC phenotype and calcification using an in vitro model of calcification.

Methods and Results—In situ hybridization of human atherosclerotic plaques (n=10) collected from patients undergoing carotid endarterectomy demonstrated that subsets of lipid-filled VSMCs adjacent to sites of calcification expressed alkaline phosphatase, bone Gla protein, and bone sialoprotein, suggesting an osteogenic phenotype. Treatment of VSMCs in culture with acetylated low-density lipoprotein (acLDL) or lipoprotein-deficient serum altered the time course of bone-associated protein gene expression and calcification. AcLDL increased nodule calcification 3-fold, whereas lipoprotein-deficient serum significantly inhibited it. Reverse transcriptase–polymerase chain reaction and Western analysis demonstrated the presence of the acLDL receptor, SRA1, exclusively in calcifying nodular VSMCs, and blockade of SRA with polyinosinic acid inhibited acLDL-induced calcification. Because apoptotic bodies can serve as nucleation sites for calcification, we investigated whether acLDL could stimulate apoptosis in nodules. Apoptosis of nodular VSMCs was unaltered, but the number of apoptotic bodies per nodule increased ~3-fold, implying a defect in phagocytosis. Consistent with these observations, binding of apoptotic bodies to VSMCs was decreased in the presence of acLDL.

Conclusions—These studies suggest that modified lipoproteins stimulate calcification by enhancing osteogenic differentiation of VSMCs and by a novel mechanism whereby acLDL interacts with SRA on VSMCs and blocks phagocytic removal of apoptotic bodies. (Circulation. 2002;106:3044-3050.)

Key Words: lipoproteins ■ apoptosis ■ muscle, smooth

Vascular calcification is a common and clinically significant component of several human diseases, including atherosclerosis, aortic stenosis, and diabetes. Its presence correlates with an increased risk of myocardial infarction,1,2 and it impedes vascular function by decreasing vessel elasticity.3 Atherosclerotic calcification is seen as early as the second decade of life, just after the fatty streak stage,4 and many studies have demonstrated a clear association between lipid accumulation and atherosclerotic calcification.4–7 Calcification colocalizes with cholesterol crystals in human atherosclerosis5 and is present in the intima of cholesterol-fed rabbits6 and ApoE knockout mice.7 Microscopic calcification in aortic valves seems to form in areas in which lipoproteins have been retained,8 and early deposits of calcification occur in and around isolated vascular smooth muscle cells (VSMCs) within the lipid core.4 Despite these associations, little is known of the role lipids play in regulating vascular calcification.

Our present understanding of the sequence of events leading to calcium crystal formation firstly involves the generation of nucleation sites. In cartilage and bone, these initiation sites are thought to be matrix vesicles, which are membrane-bound bodies, often generated in association with apoptosis, which bud off chondrocytes and osteoblasts.9,10 Similar structures derived from apoptotic VSMCs have been identified in human calcified arteries.11 Like matrix vesicles, apoptotic bodies can accumulate calcium ions and calcify in vitro,12,13 suggesting that they may be key sites for calcium crystal nucleation in plaques.

Calcification also requires the presence of a permissive matrix that allows proliferation of calcium crystals. In the normal uncalcified media, VSMCs have a contractile phenotype and constitutively express proteins that inhibit mineralization. These include matrix Gla protein (MGP),14 osteonectin, and osteopontin (OP).15 However, in calcified atherosclerotic plaques, other bone-associated proteins have been detected, including bone morphogenetic proteins, bone sialoprotein (BSP), alkaline phosphatase (ALK), and bone Gla protein (BGP).16,17 The exact role of
these induced proteins in regulating mineralization is not known, but their presence implies that osteogenic conversion of VSMCs may occur in calcified vessels. Indeed, extensive evidence in vitro has shown that VSMCs take on an osteo/chondrocytic phenotype. In this study we demonstrate for the first time the presence of subsets of VSMCs exhibiting an osteogenic gene expression profile within atherosclerotic plaques. These VSMCs were located in lipid-rich areas of the plaque, and studies in vitro demonstrated that spontaneous lipid accumulation occurred in osteogenic VSMCs and preceded their calcification. Moreover, addition or removal of lipoproteins altered the time course of osteogenic differentiation and calcification of VSMCs. Additional analysis of the mechanisms involved in accelerated calcification revealed a novel mechanism whereby modified lipoproteins act via the scavenger receptor SRA1 to inhibit VSMC phagocytosis of apoptotic bodies, thereby creating additional nidi for calcification.

Figure 1. In situ hybridization showing expression of ALK, BSP, and BGP in human carotid endarterectomy sections. A, An area of the media at the base of a plaque. m indicates the medial area; i, the intima. a through f are adjacent sections showing α-smooth muscle-actin staining (a), von Kossa staining (b), ORO staining (c), dark-field photomicroscopy demonstrating hybridization of antisense probes to ALK (d), BSP (e), and BGP (f). B, a through d are adjacent sections of an enlarged area of isolated VSMCs in the intima from panel A. Arrows indicate VSMC outline. a, α-smooth muscle-actin; b, lack of macrophages in this area by staining for CD68; c, von Kossa staining; and d, ORO staining. e through h are isolated lipid-filled intimal VSMCs showing mRNA expression for ALK (e), BSP (f), and BGP (g) (indicated by arrows). h, Negative control for in situ hybridization.

Methods

Cell Culture and Materials
VSMCs were cultured from explants of human aorta (from males ranging in age from 15 to 70 years) in 20% FCS/Medium 199, as described previously. Lipoprotein-deficient serum (LPDS) and low-density lipoprotein (LDL) were a gift from Dr C. Fitzsimmons, Cambridge, and were prepared by ultracentrifugation. Acetylated LDL (acLDL) was prepared by addition of 1.5 μL acetic anhydride per milligram of LDL in 50% ice-cold saturated sodium acetate over a 1-hour period at 4°C. The acLDL was dialyzed against PBS/0.3 mol/L EDTA and once against PBS and gave a single band on agarose electrophoresis with an REM value of 5.0, ie, pure, fully acetylated LDL.

In Situ Hybridization
Carotid endarterectomy samples from 10 patients were snap-frozen and mounted in Tissue-Tek OCT embedding compound (Miles Ames Division, Inc). Sections (8 to 10 μm) were mounted onto gelatinized slides and processed for in situ hybridization as previously described. Sense and antisense 35S-labeled cRNA probes were transcribed from cRNA of human ALK, BSP, and BGP, cloned into PCRII (Invitrogen). Slides were exposed for 3 weeks, devel-
opened, stained with H&E, and mounted. Immunohistochemistry was performed with an avidin-biotin immunoperoxidase kit (Dako). VSMCs were identified with a mouse monoclonal antibody (Dako M815, dilution 1:25) to human smooth muscle actin, and macrophages were identified with a mouse monoclonal antibody to CD68 (Dako macrophage EMB11, dilution 1:20). Calcification and lipid were demonstrated by von Kossa and oil red O (ORO) staining, respectively.

Detection of Lipid
VSMC cultures were fixed in 4% formaldehyde in PBS for 15 minutes and stained with ORO solution. Alternatively, cells were incubated with Nile red (500 ng/mL in PBS) for 30 minutes, rinsed briefly with bisbenzimide (Hoechst, 2 μg/mL in PBS, Sigma), and washed in PBS before observation with a Leica TCS-SP-MP confocal microscope. DiI-labeled acLDL (Biomedical Technologies, Inc) uptake by nodules was assessed using the manufacturer’s protocol.

Measurement of Calcification
Calcification was measured in cultures at day 28 either by staining with Alizarin red or by the cresolphthalein method as described previously.13

Reverse-Transcription Polymerase Chain Reaction
RNA and cDNA were prepared from monolayer and nodular VSMCs and analyzed by reverse-transcription polymerase chain reaction (RT-PCR) as described previously.15,18 Briefly, to ensure that each PCR reaction was performed within the linear range of amplification, test reactions were performed for each primer pair at 20, 25, 30, 35, and 40 cycles. Southern blots were performed on these reaction products and hybridized and counted. Plots of these were used to establish linearity and final quantitation, and normalization of PCR products was performed in real time using an Instant Imager.15 The oligonucleotide primers were designed from published human sequences in the Genbank/European Molecular Biology databases, with the size of the product and sequencing verifying correct gene amplification.

Western Analysis
Nodules were separated from monolayers by trypsinization and subsequent filtration through a 70-μm sieve (Falcon), which retained the nodules. Monolayer, nodules, or human peripheral blood monocytes (prepared as described previously18) were lysed, resuspended in sample buffer containing 100 mmol/L β-mercaptoethanol, and resolved by 6% SDS-PAGE after the protocol of Gough et al.20 The mouse polyclonal SRA antibody (a gift from D. Greaves, Oxford) was used at a dilution of 1:2000, and binding was detected with a peroxidase-conjugated anti-mouse IgG (Amersham, 1:1000) and visualized by chemiluminescence (ECL, Amersham).

Apoptosis/Apoptotic Body Measurement
Apoptosis was measured in nodules as described previously.13 Apoptotic bodies were quantified by counting small, intensely stained nuclei.
Hoechst-stained structures in nodules by acquiring serial optical sections through whole nodules using confocal microscopy.

**Apoptotic Body Binding Assay**

The number of apoptotic bodies bound to VSMCs was assessed by a modified method of Bennett et al.\(^\text{21}\) Briefly, VSMCs were dispersed from nodules as described previously\(^\text{18}\) and plated in 4-well chamber slides at 10,000 cells/well. Apoptotic bodies were prepared by serum-starving HASMC\(^\text{66}\) cells\(^\text{13}\) and collected by centrifugation at 2,500 rpm. The bodies were incubated with 2 g/mL Hoechst and washed with medium before incubating with VSMCs in chamber slides at 200,000 bodies/well for 2 hours at 37°C. The cells were then washed 3 times in PBS and fixed in 4% formaldehyde. Apoptotic body binding was measured by counting fluorescent bodies bound to underlying VSMCs. Approximately 50 VSMCs were counted from a random area of at least 8 different wells. In some experiments, cells were incubated with a goat SRA polyclonal antibody (Chemicon).

**Statistical Analysis**

ANOVA was used to test for differences within groups of samples. Student’s t test was used to compare two means. Results are expressed as mean±SD.

**Results**

**VSMC Expression of Osteogenic Markers in Atherosclerotic Plaques**

In situ hybridization of human carotid atherosclerotic plaques demonstrated that VSMCs in lipid-rich, calcified regions at the base of plaques expressed BSP, BGP, and ALK (Figure 1A). These genes were not expressed by normal medial VSMCs (data not shown\(^\text{17}\)). In addition, isolated, lipid-filled VSMCs in the intima also coexpressed these bone markers (Figure 1B). Thus, human VSMCs in lipid-rich atherosclerotic lesions undergo osteogenic differentiation and elaborate a bone-like matrix.

**Lipid Is Associated With VSMC Calcification In Vitro**

In vitro, human VSMCs spontaneously express bone-associated proteins and form nodules that calcify at \(\sim 28\) days.\(^\text{15,18}\) Using ORO, we demonstrated that these nodules accumulated lipid, even at early stages before calcification occurred (Figure 2A). Confocal microscopy using Nile red to image lipid within nodules revealed that the lipid droplets were present both intracellularly and extracellularly (Figure 2B).

**Requirement of Lipid/Lipoproteins for Calcification**

To test whether lipoproteins were required for calcification, VSMCs were incubated in medium containing LPDS for 28 days. The nodules that formed in the presence of LPDS stained less intensely with ORO and Alizarin red compared with native LDL controls (Figure 3A). LPDS also significantly inhibited nodule formation (control, 22.5±4.2 nodules/well; LPDS, 2.4±0.4 nodules/well; \(n=4; P<0.001\)) and calcification (Figure 3B). Conversely, addition of lipid in the form of acLDL stimulated calcification 3-fold (Figure 3B) and nodule formation (control, 22.5±4.2 nodules/well; acLDL, 37.1±4.3 nodules/well; \(n=4, P=0.003\)). Calcification and lipid accumulation were confined to the nodules after acLDL treatment; the monolayer cells did not accumulate lipid (Figure 3A). Thus, lipoproteins (or lipoprotein-associated molecules) are required for nodule formation and calcification, and this process is stimulated by acLDL.

**Mechanism of VSMC Nodule Lipid Accumulation**

We next compared the monolayer and nodular VSMCs for expression of lipoprotein and scavenger receptors using RT-PCR. Monolayer and nodular cells expressed similar levels of CLA1, CD68, LDL receptor, LDL–related protein receptor, and the very low-density lipoprotein receptor (Figure 4A), whereas SRAII, as well as CD36 and LOX-1, were not detected (data not shown). However, scavenger receptor class AI (SRAI) mRNA was absent in monolayer cells, but its expression increased as nodules developed (Figure 4A). This phenomenon was confirmed by Western analysis (Figure 4B).

**Effects of Blocking SRA on Calcification**

To test the involvement of SRAI in calcification, VSMC cultures were incubated with acLDL in the presence of...
polyinosinic acid (PI), a ligand for SRA. PI was found to
block the stimulatory effect of acLDL on nodule calcification
(Figure 5A). To confirm the interaction of PI with SRA,
nodules were incubated with or without PI and assessed for
acLDL uptake using DiI-acLDL. Nodules incubated with PI
accumulated less DiI-acLDL (Figure 5B).

Effects of acLDL on Osteogenic Differentiation
of VSMCs
Human VSMCs were treated with either LPDS, native LDL,
or acLDL for up to 28 days, and expression of osteogenic
markers was assessed by RT-PCR (Figure 6). As previously
reported, human VSMCs default to an osteo/chondrocytic
phenotype in vitro, and their expression of bone markers
changes as calcification occurs. Compared with native
LDL-treated controls, LPDS-treated cells expressed higher
levels of OP and BGP. Treatment with acLDL increased
expression of ALK and BSP by day 21. AcLDL and LPDS
did not alter the expression of MGP or osteonectin mRNA
(data not shown). Therefore, lipoproteins influence the
expression of a subset of bone marker genes by VSMCs in vitro.

Effects of acLDL on Apoptosis
Nodules treated with acLDL had similar apoptotic indices to
native LDL controls (Figure 7A). However, there was a
greater abundance of apoptotic bodies within nodules treated
with acLDL (Figure 7B). This implied that acLDL might
interfere with phagocytic removal of apoptotic bodies. This
was tested using an apoptotic body binding assay in which
apoptotic bodies derived from VSMCs were labeled with
Hoechst and incubated with SRA1-positive VSMCs dis-
persed from nodules (Figure 8A). This assay showed that
VSMC-apoptotic body binding was significantly reduced in
the presence of either acLDL or SRA antibodies (Figures 8B
and 8C). AcLDL had no effect on apoptotic body binding to
SRA1-negative monolayer VSMCs (data not shown).

Discussion
Previous studies have demonstrated a link between lipids and
vascular calcification and implied that lipids may have a role
in nucleating calcium crystals or stimulating osteogenic
differentiation. We have extended these studies in human
VSMCs and investigated the expression of several bone
marker genes as well as the role of lipoproteins in apoptosis
and apoptotic body clearance. This study demonstrates, for
the first time, that an osteogenic phenotype of VSMCs exists
in atherosclerotic lesions in association with sites of lipid
accumulation and calcification.

The mechanisms linking lipid, osteogenic gene expression,
and calcification observed in vivo were investigated using a
model of VSMC calcification where postconfluent VSMCs
form nodules that calcify within 28 days. These studies
demonstrated that lipid accumulation occurred spontaneously
in nodules and in the absence of lipoproteins, fewer nodules
formed, less lipid accumulated, and less calcification oc-

curred. In the presence of acLDL, nodule formation, lipid
accumulation, and calcification were stimulated above the
native LDL controls. The mechanism by which lipid accu-
mulates in VSMCs is not known, but we showed that human
VSMCs and nodules expressed several lipoprotein and scav-
enger receptors. In particular, the acLDL receptor, SRA1,
which is not normally expressed by cultured VSMCs, was
specifically induced in nodular cells. These studies raised the
question of why SRA1 is induced in VSMCs, and one
possibility is that in the apoptotic environment of the VSMC
node, oxidative stress would be expected, which is a known
inducer of expression of SRA in VSMCs. Treatment of
nodules with PI inhibited acLDL-induced stimulation of
calcification. Although PI is not a specific SRA ligand, these
results suggest that acLDL may interact with SRA1 to
stimulate calcification.

Treatment of VSMC cultures with LPDS or acLDL
changed their osteogenic gene expression profile. However,
the extent to which changes in bone-marker gene expression
affect calcification is not clear because of the poor knowledge
of the function of many of the proteins involved. ALK and
BSP are thought to be procalcification factors, whereas BGP
and OP have been demonstrated as anticalcification fac-
tors. VSMCs exposed to LPDS expressed higher levels of
BGP and OP mRNA than calcifying nodular cultures, in
keeping with the inhibitory action of these proteins. Con-
versely, acLDL increased expression of ALK and BSP, which might stimulate nodular calcification. Indeed, increased expression of ALK in nodular cells by acLDL could potentially stimulate production of apoptotic bodies with increased ALK activity, which is known to be a key regulator/initiator of matrix vesicle calcification via generation of local phosphate ions.9 These findings are supported by other studies in which minimally oxidized LDL and isoprostanes stimulated ALK activity in calcifying vascular cells.24

Our previous studies have shown that apoptosis occurs spontaneously at a relatively high rate in nodules (≈20%) and that alteration of the apoptotic rate influences calcification.13 These studies also showed that apoptotic bodies derived from VSMCs can serve as a nidus for calcification. Apoptotic bodies are normally rapidly phagocytosed in vivo, but their detection in atherosclerotic plaques10,11 implies that phagocytosis is inhibited, which would promote calcification in an environment with sufficient local Ca$^{2+}$, PO$_4^{3-}$, and appropriate matrix. Given the importance of apoptosis in calcification and because other studies have demonstrated that modified lipoproteins can stimulate apoptosis,28 we tested the effects of acLDL on nodule apoptosis. There was, however, no effect of acLDL on nodule apoptosis, but we did find a greater number of apoptotic bodies in acLDL-treated nodules. This implied a defect in phagocytosis of apoptotic bodies by nodular VSMCs in the presence of acLDL, which was confirmed in an apoptotic body binding assay. SRA antibodies also inhibited apoptotic body binding, which provided a mechanism to explain how acLDL might stimulate calcification; ie, acLDL competes with apoptotic bodies for SRA binding. Other studies have shown that oxidized LDL competes with apoptotic bodies for macrophage binding,29 and SRA is thought to be important in this process.30 This raises the possibility that other oxidized lipids that are known to stimulate calcification could also act via inhibition of phagocytosis. Thus, our studies suggest that VSMC as well as macrophage phagocytosis is likely to be inhibited in the setting of the lipid-rich atherosclerotic plaque and may explain why apoptotic bodies are detected in these plaques.31 In relation to this, a lack of phagocytic function in plaques may also contribute to the finding of vascular-derived apoptotic vesicles in circulating blood of atherosclerosis-prone patients.32

In conclusion, these studies highlight a new role for lipoproteins and VSMCs in the development of calcification in atherosclerosis.

Acknowledgments
This study was supported by a British Heart Foundation program grant (RG/200004).


Figure 8. A, Demonstration of apoptotic body binding to VSMCs. VSMC-derived apoptotic bodies were labeled with Hoechst and incubated with VSMCs dispersed from nodules for 2 hours. After extensive washing with PBS and fixing, the cultures were analyzed by fluorescence microscopy. AcLDL 100 μg/ml (B) and SRA polyclonal antibody 1:200 (C) significantly inhibited the number of apoptotic bodies bound to VSMCs (*P<0.0002, n=12; †P<0.0001; n=8, respectively).

References


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_Circulation_. 2002;106:3044-3050; originally published online November 11, 2002; doi: 10.1161/01.CIR.0000041429.83465.41
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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