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

D. Proudfoot, PhD; J.D. Davies, PhD; J.N. Skepper, PhD; P.L. Weissberg, MD, FRCP; C.M. Shanahan, PhD

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 = 110) 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 atherosclerosis8 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.16–18

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.

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.18 Lipoprotein-deficient serum (LPDS) and low-density lipoprotein (LDL) were a gift from Dr C. Fitzsimmons, Cambridge, and were prepared by ultracentrifugation.19 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.15 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 \(<s/>\text{H}9251\text{-smooth muscle actin, and macrophages were identified with a mouse monoclonal antibody to \text{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 \(\mu\)g/mL in PBS, Sigma), and washed in PBS before observation with a Leica TCS-SP-MP confocal microscope. DiI-labeled acLDL (DiI-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- \(\mu\)m 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 \(\beta\)-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, Oxford20) 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

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**Figure 2.** Lipid staining in 7-day VSMC nodular cultures. A, ORO staining revealed that lipid was only associated with the nodule, not the monolayer. B, A maximum intensity projection of a whole nodule incubated with Nile red (shown as green) and Hoechst (blue) generated by confocal microscopy. The small arrow indicates intracellular lipid droplets, whereas the larger arrow indicates a pool of extracellular lipid.

**Figure 3.** Analysis of lipid and calcification in 28-day nodules treated with 20% LPDS, native LDL (\(n\)LDL, 100 \(\mu\)g/mL), or acLDL (100 \(\mu\)g/mL). A, ORO and Alizarin red staining are much weaker in LPDS-treated nodules compared with either \(n\)LDL- or acLDL-treated nodules. B, Measurement of calcification using the cresolphthalein method. Controls were incubated with 20% FCS/M199 alone.
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. The number of apoptotic bodies bound to VSMCs was assessed by a modified method of Bennett et al.21 Briefly, VSMCs were dispersed from nodules as described previously and plated in 4-well chamber slides at 10,000 cells/well. Apoptotic bodies were prepared by serum-starving HASMC cells and collected by centrifugation at 2500 rpm. The bodies were incubated with 2 μg/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). 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 ~28 days. 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 SRAI, 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 SRA1 in calcification, VSMC cultures were incubated with acLDL in the presence of...
stained nodules. The lower panel shows corresponding Hoechst- fluorescence microscopy. Uptake of DiI-acLDL was reduced in the fl

Figure 5. A, Effect of polyinosinic acid (PI) on acLDL-induced calcification; VSMCs were treated with either acLDL (100 \(\mu\)g/mL), PI (100 \(\mu\)g/mL), acLDL + PI, or no additions (control) for 28 days and calcification measured by counting numbers of nodules positive for Alizarin red staining. PI had no effect alone but significantly inhibited the acLDL-induced calcification. B, Effect of PI on acLDL uptake into nodules. Dil-acLDL (10 \(\mu\)g/mL) was incubated with nodular cultures \(\pm\) PI (100 \(\mu\)g/mL) for 4 hours, and uptake was qualitatively estimated using fluorescence microscopy. Uptake of Dil-acLDL was reduced in the presence of PI. The lower panel shows corresponding Hoechst-stained nodules.

polynucleobasic 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 Dil-acLDL. Nodules incubated with PI accumulated less Dil-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.\(^{17}\) 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 dispersed 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\(^{5,22}\) or stimulating osteogenic differentiation.\(^{23,24}\) 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 occurred. In the presence of acLDL, nodule formation, lipid accumulation, and calcification were stimulated above the native LDL controls. The mechanism by which lipid accumulates in VSMCs is not known, but we showed that human VSMCs and nodules expressed several lipoprotein and scavenger receptors. In particular, the acLDL receptor, SRA1, which is not normally expressed by cultured VSMCs, was specifically induced in nodular cells. These studies raise the question of why SRA1 is induced in VSMCs, and one possibility is that in the apoptotic environment of the VSMC nodule, oxidative stress would be expected, which is a known inducer of expression of SRA in VSMCs.\(^{25}\) 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 factors.\(^{26,27}\) 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. These findings are supported by other studies in which minimally oxidized LDL and isoprostanes stimulated ALK activity in calcifying vascular cells. 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. 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 plaques implies that phagocytosis is inhibited, which would promote calcification in an environment with sufficient local calcium and appropriate matrix. Given the importance of apoptosis in calcification and because other studies have demonstrated that modified lipoproteins can stimulate apoptosis, 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, and SRA is thought to be important in this process. 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. 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.

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

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P0.0002, n/H1102112; *0.0001; n(*/H11021/H11005
inhibited the number of apoptotic bodies bound to VSMCs
Figure 8.
A, Demonstration of apoptotic body binding to
features were analyzed by
2 hours. After extensive washing with PBS and incubated with VSMCs dispersed from nodules for
nLDL acLDL
Number of apoptotic bodies bound/cell
Control serum Anti-SRA

References
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