Oxidized Low-Density Lipoprotein and Upregulated Expression of Osteonectin and Bone Sialoprotein in Vascular Smooth Muscle Cells

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ABSTRACT

Background: Oxidative stress has been associated with the progression of atherosclerosis and activation of genes that lead to increased deposition of proteins in the extracellular matrix. Bone sialoprotein (BSP) and osteonectin are proteins involved in the initiation and progression of vascular calcification.

Objective: To investigate the effect of oxidized low-density lipoprotein on osteonectin and BSP expression in human aorta vascular smooth muscle cells (HA/VSMCs).

Methods: We treated HA/VSMCs with oxidized low-density lipoprotein (oxLDL) and measured the relative expression of osteonectin and BSP genes using the real-time polymerase chain reaction (PCR) method. We investigated the protein levels produced by each gene using the western blotting technique.

Results: oxLDL increased osteonectin and BSP levels (mean [SD], 9.1 [2.1]–fold and 4.2 [0.75]–fold, respectively) after 48 hours. The western blotting results also confirmed the increased levels of osteonectin and BSP.

Conclusion: oxLDL may enhance vascular calcification by promoting the expression of osteonectin and BSP.

Keywords: atherosclerosis, vascular calcification, oxidized low-density lipoprotein, bone sialoprotein, osteonectin, smooth muscle cells

Cardiovascular disease is the most common cause of death in industrialized countries. Atherosclerosis is characterized by lesions in the arterial intima leading to narrowing of the vessel lumen.[1] *Vascular calcification*, the pathologic deposition of calcium phosphate in blood vessels and valves, is generally observed in atherosclerosis.[2] Oxidative stress activates genes that lead to increased deposition of extracellular matrix proteins,[3] which are an important factor in the progression of vascular calcification.[4] Accumulation of oxidized low-density lipoprotein (oxLDL) in the vascular wall stimulates the development of atherosclerosis and vascular calcification.[5]

Osteonectin is a calcium-binding protein involved in bone formation[6] that binds to hydroxyapatite.[7] Osteonectin is expressed by the cells in the vascular wall, specifically during calcification of atherosclerotic plaques.[8] This protein plays an important role in atherogenesis and may be a biomarker of atherosclerosis and calcinosis in coronary arteries.[9] Bone sialoprotein (BSP) also is involved in the initiation of atherosclerosis. Microarray analysis reveals that BSP is overexpressed in human carotid plaques.[10]

Although the role of oxLDL in the progression of atherosclerosis has been suggested in the literature, little is known about the effect of oxLDL on extracellular matrix proteins involved in vascular calcification. Hence, research on the cause of vascular calcification may be useful in preventing atherosclerosis. In this study, we examined the ability of oxLDL to induce osteonectin and BSP, 2 extracellular matrix proteins involved in intravascular mineralization.

Abbreviations

oxLDL, oxidized low-density lipoprotein; BSP, bone sialoprotein; HA/VSMCs, human aorta vascular smooth muscle cells; cDNA, complementary DNA; RT-PCR, real-time polymerase chain reaction; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay; A280, absorbance level of 280 nm; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; IgG, immunoglobulin G; LDL, low-density lipoprotein

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Table 1. Primer Sequences and Product Length

Gene	Primer Sequences (5'-3')	Product Length, bp
Osteonectin[a]		
Forward	TCTTCCCTGTACACTGGCAGTTC	73
Reverse	AGCTCGGTGTGGGAGAGGTA	
BSP[b]		
Forward	TGCCTTGAGCCTGCTTCCT	79
Reverse	CTGAGCAAAATTAAAGCAGTCTTCA	
GAPDH[c]		
Forward	ACACCCACTCCTCCACCTTTG	112
Reverse	TCCACCACCCTGTTGCTGTAG	

BSP, bone sialoprotein; GADPH, Glyceraldehyde 3-phosphate dehydrogenase.
[a] *NM_003118.3*
[b] *NM_004967.3*
[c] *NM_002046.5*

Materials and Methods

Cell Culture and Treatment

We purchased human aorta vascular smooth muscle cells (HA/VSMCs) from the Pasteur Institute of Iran, Tehran. We maintained VSMCs in F12K media containing 0.05 mg/mL ascorbic acid; 0.01 mg/mL insulin; 0.01 mg/mL transferrin; 10 ng/mL sodium selenite; 0.03 mg/mL endothelial cell growth supplement; fetal bovine serum to a final concentration of 10%; 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES] to a final concentration of 10 mmol; tetradecyl sulfate, ethanol, and saline (TES) to a final concentration of 10 mmol; 100 U/mL penicillin; 100 U/mL streptomycin; and 0.01% amphotericin B. Cells were incubated in a humidified atmosphere of 5% CO2 to 95% air at 37°C. The cells that we used underwent 3 to 7 passages and VSMC calcification was induced by the presence of 10 mmol β-glycerophosphate.

We seeded HA/VSMCs into a 12-well plate at a density of approximately 10,000 cells per well. When the cells achieved approximately 80% confluence, we changed the media to media containing 80 µg/mL oxLDL; Cells were incubated for 24 and 48 hours. Control cells were cultured in media containing β-glycerophosphate without oxLDL.

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA from the cell line was extracted using Biozol extraction fluid (BIOZOL Total RNA Extraction Reagent, BioFlux, Japan). We reverse transcribed a total of 0.3 µg of total RNA using the complementary DNA (cDNA) synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA). Real-time PCR (RT-PCR) was performed using the RotoGene system (Corbett Research, Sydney, Australia) and the SYBR green (Thermo Fisher Scientific Inc.) methods using primers (Table 1) for osteonectin and BSP genes (NM_003118.3 and NM_004967.3, respectively). Products were amplified as follows: initial enzyme activation at 94°C for 5 minutes, then 40 cycles of 95°C for 15 seconds, 59°C for 20 seconds, and 72°C for 30 seconds. Quantitation of data was by the comparative CT (ΔΔCT) method that uses expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (NM_002046.5) as an endogenous reference.

Western Blot Analysis

For western blotting from cell culture, we washed cells twice with cold phosphate-buffered saline (PBS) and lysed them in ice-cold 6× radioimmune precipitation assay (RIPA) buffer. We incubated the homogenate in lysis buffer for 30 minutes and then centrifuged it at 12,000 g for 10 minutes at 4°C. Supernatants were collected as whole-cell lysates for western blotting. We measured protein concentration using a spectrophotometric method (NanoDrop 2000, Thermo Fisher Scientific) at A_{280}. Equal amounts of protein from cell lysates were mixed with Laemmli buffer, boiled for 5 minutes, and separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred to polyvinylidene difluoride (PVDF) membranes in Tris-glycine buffer for 2 hours at 120 Volts (V). We blocked the membranes with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween-20 tryptic soy broth (Aurion, Wageningen, Netherlands) overnight at 4°C. The membranes were incubated with PBS, rabbit antiosteonectin polyclonal antibody (2 µg/mL) (Abcam Plc, Cambridge, England) and anti-BSP antibody (1 µg/mL) (Abcam Plc) for 2 hours at room temperature. After washing, the membranes were incubated with goat anti–rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (Abcam Plc) diluted 1:10,000 for 90 minutes at room temperature. Finally, we incubated the membranes with 3, 3′-5, 5′-Tetramethylbenzidine (BM Blue, F. Hoffman–La Roche, Ltd., Basel, Switzerland) and peroxidase substrate solution at room temperature until the developed bands were of desired intensity. Cell lysates were detected on a separate membrane with actin as a loading control.

Statistical Analysis

Statistical analysis included the nonparametric Kruskal-Wallis test and pair-wise comparisons among groups

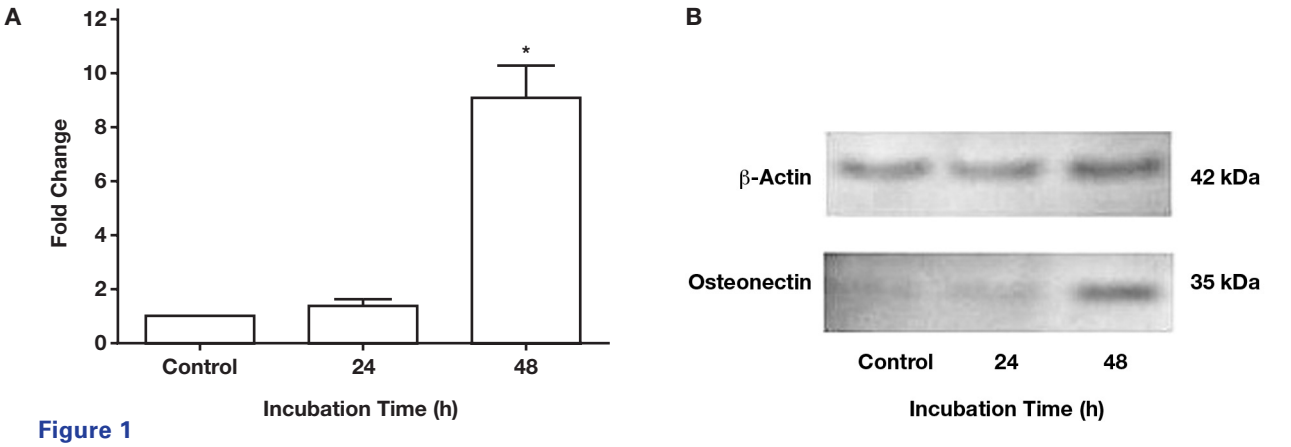

Figure 1

The effect of oxidized low-density lipoprotein (oxLDL) on osteonectin expression in human aorta vascular smooth muscle cells (HA/VSMCs). **A**, Osteonectin messenger RNA (mRNA) expression compared with the control material after treatment with oxLDL using real-time polymerase chain reaction. Data are expressed as mean (SD) for the 3 independent experiments. The * indicates $P < .05$. **B**, Results of protein expression levels in VSMCs by western blot analysis against control (β-actin) protein after treatment.

via the Mann-Whitney test. We performed all statistical analyses using Graph Pad Prism5 software (GraphPad Software, Inc., La Jolla, CA). We considered $P < .05$ to be significant.

Results

Effect of oxLDL on Osteonectin Expression

After treatment of VSMCs with 80 µg/ml oxLDL, osteonectin expression at 24 hours and 48 hours was increased (mean [SD], 1.4 [0.3]–fold and 9.1 [2.1]–fold, respectively) compared with the control group. The oxLDL had no significant effect on osteonectin gene expression after 24 hours; however, we observed a significant increase after 48 hours of treatment ($P < .05$).

The messenger-RNA (mRNA) level (**Figure 1A**) and protein-expression level (**Figure 1B**) of osteonectin showed a time-dependent increase after oxLDL treatment. Also, treatment of HA/VSMC with oxLDL yielded 35-kDa protein after 48 hours (**Figure 1B**). These results show that oxLDL upregulated the expression of the osteonectin gene.

Effect of oxLDL on BSP Expression

After treating VSMCs with 80 µg/ml oxLDL, we detected the mRNA- and protein-expression levels of BSP via quantitative RT-PCR and western blot assay (**Figure 2**). The result showed that oxLDL increased BSP expression (mean [SD], 1.5 [0.39]–fold and 4.2 [0.75]–fold after 24 and 48 hours, respectively. Overall treatment after 48 hours was significant ($P < .05$) (**Figure 2**). Beta-actin (42 kDa) was used as an internal control to standardize the protein loading in western blotting. These results suggest that oxLDL may promote BSP upregulation in VSMCs and therefore could have an effect on vascular calcification.

Discussion

It is clear that atherosclerotic calcification is a regulated process that is similar to bone formation. Many bone-related proteins are involved in this process. Also, the results of previous studies[11,12] have shown the role of oxLDL in VSMC mineralization. In addition, 2 studies[13,14] have demonstrated localization of osteoblast-differentiation factors in calcified atherosclerotic lesions.

VSMCs have a contractile phenotype and express proteins that inhibit mineralization. However, in atherosclerosis, a transition of VSMCs toward the osteoblastic phenotype occurs, and these cells express promoters of calcification including osteonectin, osteocalcin, alkaline phosphatase, and BSP. An increase in the factors that promote calcification and a decrease in the inhibitory factors cause atherosclerosis to occur.[15]

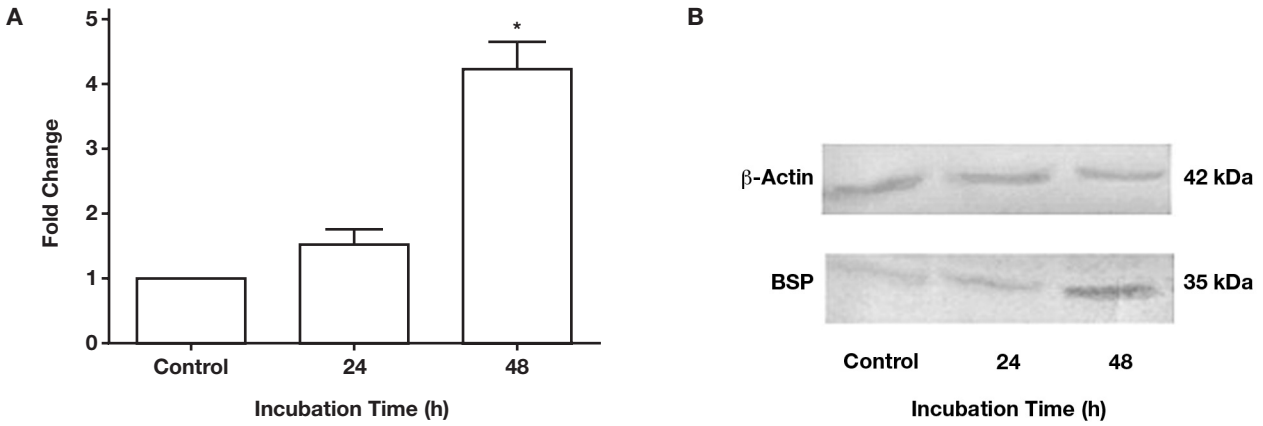

Figure 2

The effect of oxidized low-density lipoprotein (oxLDL) on bone sialoprotein (BSP) expression in human aorta vascular smooth muscle cells (HA/VSMCs). **A**, BSP messenger RNA (mRNA) expression against control after treatment with oxLDL using real-time polymerase chain reaction. Data are expressed as mean (SD) of 3 independent experiments. The * indicates P <.05. **B**, Results of protein expression levels in VSMCs by western blot analysis against control (ß-actin) protein after treatment.

In the present study, we show that oxLDL increases the expression of osteonectin, which is a promoter of calcification. Another study[16] has also shown overexpression of osteopontin and alkaline phosphatase in response to oxidative stress. Ragino et al[9] showed that osteonectin was correlated with certain key biomarkers of atherosclerosis, such as oxLDL, and suggested that osteonectin can serve as a new biomarker of atherosclerosis and calcinosis of coronary arteries.

In our study, oxLDL increased BSP expression, suggesting that BSP is involved in vascular calcification. Another study[17] also reported that acetylated low-density lipoprotein (LDL) increased expression of alkaline phosphatase and BSP. The results of this study may suggest that modified lipoproteins stimulate calcification by enhancing osteogenic differentiation of VSMCs.[17]

In contrast, the results of a study[18] suggest that BSP is involved in the inhibition of arterial calcification;[18] however, the potential of BSP to nucleate hydroxyapatite suggests that BSP may act as activator of calcification.[19,20] BSP can act as a nucleator of crystal formation. Nevertheless, the ability of bone-related proteins to promote or inhibit calcification depends on the environment of the expression,[21] which suggests that BSP may act as activator of calcification.

Because oxidative stress plays an important role in the development of atherosclerosis and the induction of vascular calcification,[5,22] understanding its mechanism of action may help prevent the development of atherosclerosis. Antioxidant therapy might have beneficial effects on the reduction of vascular calcification.

The results of this study establish a direct connection between bone-related protein expression and oxLDL in the development of calcification in VSMCs, and the role of oxLDL in promotion of calcification, and foam-cell formation in atherosclerosis. Future studies are needed to clarify the molecular mechanisms that induce the expression of the BSP and osteonectin.

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