Oxidized Low-Density Lipoprotein Increases Bone Sialoprotein Expression in Vascular Smooth Muscle Cells Via Runt-Related Transcription Factor 2

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Abstract: *Background:* Vascular calcification is a pivotal stage in atherosclerosis. During vascular calcification, vascular smooth muscle cells (VSMCs) synthesize many osteogenic factors such as bone sialoprotein (BSP). Oxidative stress plays a critical role in progression of atherosclerosis and also increases extracellular matrix proteins expression. *BSP* overexpression has been observed during vascular calcification by oxidative stress. However, the regulatory mechanism of oxidized low-density lipoprotein (oxLDL)-mediated vascular calcification has not yet been fully defined. In this study, we aimed to investigate whether runt-related transcription factor 2 (Runx2) affects the oxLDL-induced *BSP* expression or not. *Methods:* In this experimental study, we cultured VSMCs in F12K media and then treated them with oxLDL. The expression of *Runx2* and *BSP* genes was determined by real-time polymerase chain reaction method. Protein level of each gene was investigated by Western blotting technique. To determine whether Runx2 regulates *BSP* gene expression at VSMCs induced by oxLDL, we suppressed Runx2 mRNA using siRNA. Transfected cells then were treated with oxLDL and expression of *Runx2* and *BSP* genes was determined again. *Results:* oxLDL increased *Runx2* and *BSP* expression (4.8 $\pm$ 0.47-fold and 4.91 $\pm$ 0.56-fold, respectively) after 48 hours. Western blotting method confirmed the increased levels of Runx2 and *BSP* proteins after 48 hours. *Runx2* overexpression alone induced *BSP* expression, whereas knockdown of *Runx2* with small interfering siRNA blocked oxLDL-induced *BSP* expression. *Conclusions:* Our results showed that oxLDL-induced *BSP* expression was dependent on *Runx2* expression, suggesting that Runx2 is required for oxLDL-induced BSP expression.

Key Indexing Terms: Vascular calcification; Oxidized low-density lipoprotein; Bone sialoprotein; Runx2. **[Am J Med Sci 2015;349 (3):240–243.]**

Vascular calcification is one of the problems in cardiovascular diseases as it reduces vascular wall elasticity and leads to heart attacks. Vascular smooth muscle cells (VSMCs) play a significant role in vascular calcification by migration, proliferation and secretion of matrix components.[1]

During vascular calcification, VSMC differentiates to osteoblast-like phenotype and synthesize many osteogenic factors, including osteocalcin, alkaline phosphatase (ALP), bone morphogenetic proteins and bone sialoprotein (BSP).[2] BSP, a bone-associated protein is involved in the initiation of atherosclerosis. It has been suggested that BSP exhibits hydroxyapatite nucleation activity[3,4] and may play important roles in the initiation and calcification of atherosclerosis. Microarray analysis reveals overexpression of *BSP* in human carotid plaques.[5]

Many factors that play a role in induction of VSMCs to osteoblast differentiation have already been identified. It has been shown that oxidized low-density lipoprotein (oxLDL) has a critical role in the pathogenesis and development of atherosclerosis[6,7] and induces osteogenic factors expression in atheroma formation.[8,9]

Runt-related transcription factor 2 (Runx2) is a key transcription factor for osteoblast differentiation and regulates the expressions of many osteogenic factors.[10,11] *In vitro* studies have demonstrated that Runx2 plays an essential role in oxidative stress-induced VSMC calcification and Runx2 alone is sufficient to induce VSMC calcification.[12] However, the potential link between BSP expression and oxidative stress-induced vascular calcification has not been examined. We hypothesize that oxLDL upregulates *BSP* expression in human VSMCs through Runx2.

MATERIALS AND METHODS

The human aorta vascular smooth muscle cells (HA/VSMCs) and F12K media were purchased from Pasteur Institute of Iran. OxLDL was purchased from Biomedical Technologies (Stoughton, MA). SYBR Green PCR Master Mix, cDNA synthesis kit and DNase were obtained from Thermo Fisher Scientific (Waltham, MA). Trizol was obtained from Invitrogen (Invitrogen, Carlsbad, California). SiRNAs, opti-MEM and lipofectanine were purchased from Invitrogen (Ambion, Austin, TX, USA). FITC (fluorescein isothiocyanate)-conjugated siRNA was obtained from Santa Cruz Biotechnology. Anti-Runx2, anti-BSP, anti-beta actin and secondary antibody were obtained from Abcam (Cambridge, United Kingdom). 3,3′,5,5′-tetramethylbenzidine (BM blue) was purchased from Roche (Mannheim, Germany).

Cell Culture, RNA Isolation and cDNA Synthesis

In this experimental study, HA/VSMC were maintained in F12K media. F12K media contained 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, FBS to a final concentration of 10%, HEPES to a final concentration of 10 mM, TES to a final concentration of 10 mM, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.01% amphotericin B. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Daily control of cell growth and cell division in culture condition were done. Cells used for the experiments were 3 to 7 passages.

To induce VSMC calcification, the cells were incubated in the presence of 10 Mm β-glycerophosphate for 12 days.

Cells were seeded in a 12-well plate at a density of 10,000 cells per well. When the cells achieved approximately

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80% confluence, they were co-cultured with oxLDL (100 μg/mL). Control cells were cultured in media containing β-glycerophosphate without oxLDL.

After 24 and 48 hours, total RNA was extracted from the cells using Trizol according to the manufacturer instructions.

RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific) and treated with DNase. Then cDNA was synthesized from 0.5 μg total RNA using random primer and the Rever Aid First Standard cDNA Synthesis kit.

Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) was performed using Corbett system and SYBR green method.

Primers used for real-time PCR are listed in Table 1. PCRs were performed in triplicate using 5 μL SYBR green PCR Master Mix, 0.2 μL primer sets, 1 μL cDNA and 3.6 μL nuclease-free H2O to yield a 10 μL reaction. The amplification was carried out as follows: initial enzyme activation at 94°C for 10 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 performed using the comparative C_T ($\Delta\Delta C_T$) method using GAPDH gene expression as an endogenous reference.

Western Blot

Cells were washed twice with cold phosphate buffer saline and lysed in ice-cold 6× radio immune precipitation assay buffer. The homogenate was incubated in lysis buffer for 30 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was used as total cell lysate. Protein concentration was measured spectrophotometrically by nanodrop and equal amounts of protein from each sample were subjected to blotting. Protein lysate was mixed with laemmli buffer, boiled for 5 minutes and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred to a polyvinylidene-difluoride membrane in tris-glycine buffer for 2 hours at 120 V.

The membrane was blocked by 5% nonfat dry milk in Tris-buffered saline, and 0.1% tween-20 overnight at 4°C temperature. Then, the membrane was incubated in Tris-buffered saline, and 0.1% tween-20 containing the primary rabbit polyclonal anti-BSP (1 μg/mL) and primary rabbit polyclonal anti-Runx2 at 1:2,000 for 2 hours at room temperature. After washing, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate diluted 1:10,000 for 90 minutes at room temperature. Finally, the color was developed with the addition of 3,3′,5,5′-tetramethylbenzidine membrane peroxidase substrate. The color reaction was stopped by washing the membranes with distilled water. Cell lysates were detected on a separate membrane with actin as a loading control.

Runx2 RNA Interference

To knockdown *Runx2*, we used 2 siRNAs (IDs: s2455 and s2456). Runx2 siRNAs, s2455 and s2456, are located in the region of the *Runx2* transcript that codes for amino acids 910 to 928 and 1,663 to 1,683 respectively. The oligonucleotide sequences were as follows: s2455: 5′-CUUGAUGACUCUAAACC UATT-3′ and 5′-UAGGUUUAGAGUCAUCAAGCT-3′, s2456: 5′-CCAAAUUUGCCUAACCAGATT-3′and 5′-UC UGGUUAGGCAAAUUUGGAT-3′.

The cells were seeded in a 24-well plate at the density of 20,000 cells per well in growth medium without antibiotics. After 24 hours, the cells were transfected with RNAi duplex—Lipofectamine RNAiMAX complexes made in Opti-MEM according to the manufacturer's instructions. A 10 nM of each siRNA was used for all transfections. siRNA transfection efficiency was observed by uptake of FITC-labeled siRNA sequence, and scramble oligoribonucleotide duplex that was not homologous to any mammalian genes was used as a control. After 24 hours, the transfection media were removed, and the cells were incubated for an additional 24 hours in normal growth media and then stimulated with oxLDL (100 μg/mL). The cells were harvested for mRNA and protein extraction after 48 hours. Because of a significant increase in *BSP* expression observed after 48 hours, Runx2 knock-down was only done at 48 hours.

Statistical Analysis

All experiments were done in triplicate. Statistical analysis was done using nonparametric Kruskal-Wallis test, and pairwise comparisons among groups were performed by Mann-Whitney's *U* test. All statistical analyses were performed with Graph Pad Prism5 software. All data were presented as mean ± standard error of mean, and $P < 0.05$ was considered as the level of significance.

RESULTS

Effect of oxLDL on *BSP* and *Runx2* Expression

After treatment of VSMCs with 100 μg/mL oxLDL, the mRNA and protein levels of Runx2 and BSP were detected by quantitative real time-PCR and Western blotting assay. The result showed that oxLDL increased *Runx2* expression (2.29 ± 0.39-fold and 4.8 ± 0.47-fold) and *BSP* expression (1.46 ± 0.45-fold and 4.91 ± 0.56-fold) after 24 and 48 hours, respectively (Figures 1A and 1B). The significant increment of *BSP* gene expression was observed only after 48 hours of treatment. Differences between groups were determined as significant at $P < 0.05$. Western blot analysis confirmed the changes observed at mRNA level (Figure 1C). In Western blot analysis, beta-actin (42 kDa) was used as internal control.

siRNA Transfection

To determine whether Runx2 regulates *BSP* gene expression at VSMCs induced by oxLDL, we suppressed Runx2 mRNA using siRNA. VSMCs were transfected with Runx2 siRNA (siRunx2) or nontargeting control siRNA and then treated with oxLDL. Transfection efficacy was determined using

TABLE 1. Primer sequences and product length

Genes	Primer sequences (5′–3′)	Product length (bp)
Runx2	Forward: CGATCTGAGATTTGTGGGCC	76
	Reverse: GGGAGGATTTGTGAAGACGG	
BSP	Forward: TGCCTTGAGCCTGCTTCCT	79
	Reverse: CTGAGCAAAATTAAAGCAGTCTTCA	
GAPDH	Forward: ACACCCACTCCTCCACCTTTG	112
	Reverse: TCCACCACCCTGTTGCTGTAG	