Protection Against Oxidized Low-Density Lipoprotein–Induced Vascular Endothelial Cell Death by Integrin-Linked Kinase

Xiuwu Zhang, MD, PhD; Kang Hu, MS; Chuan-Yuan Li, PhD

Background—Integrin-linked kinase (ILK) is a protein that plays important roles in extracellular matrix-mediated signaling. It has been shown that ILK is expressed preferentially in cardiac and skeletal muscles. Evidence points to its role as an upstream regulator of protein kinase B, a critical player in apoptosis. Because oxidized LDL (oxLDL) is thought to promote atherogenesis by causing the apoptosis of endothelial cells, we investigated the potential roles that ILK may play in oxLDL-induced apoptosis in vascular endothelial cells.

Methods and Results—Transcriptional and translational levels of ILK were investigated with reverse-transcriptase polymerase chain reaction and Western analysis. oxLDL treatment induced both the transcription and the translation of the ILK gene in endothelial cells. A recombinant adenovirus vector encoding the ILK gene was constructed to investigate its potential role in oxLDL-induced apoptosis in human umbilical vein endothelial cells and mouse lymphoid vein endothelial cells transformed by simian virus 40. In both types of cells, overexpression of the ILK gene significantly prevented oxLDL-induced apoptosis or cell death, as evaluated by 2 independent assay methods. Furthermore, we showed that ILK could inhibit oxLDL-induced upregulation of the kinase activity of p38 mitogen-activated protein kinase, which is often associated with stress-induced pro-apoptotic signal transduction. Finally, examination of other factors, such as bcl-2, bcl-xl, caspase 3, and caspase 9, demonstrated significant changes that were correlated with oxLDL treatment and ILK overexpression.

Conclusion—ILK may be an important factor involved in the regulation of oxLDL-induced apoptosis in vascular endothelial cells. Modifying its activity may be a useful approach for prevention of endothelial cell injury in oxLDL-induced atherosclerosis. **(*Circulation*. 2001;104:2762-2766.)**

Key Words: oxygen ■ lipoproteins ■ endothelium ■ apoptosis ■ kinases

Oxidized LDL (oxLDL) has been implicated in atherosclerosis in both epidemiological and laboratory studies.[1,2] The precise role that oxLDL plays in atherogenesis is unknown. There is, however, increasing evidence to suggest that oxLDL-induced injury of the vascular wall is critical because it may be the initiating event that leads to the inflammatory responses now recognized to be the main cause of atherosclerosis. How does oxLDL induce injury to the vascular wall? Many recent studies suggest that oxLDL-induced apoptosis in the vascular smooth muscle cells or vascular endothelial cells may be the key. In support of this theory, several studies have demonstrated the presence of apoptosis in human and experimental atherosclerotic plaques.[3,4] Tissue culture experiments have indicated that oxLDL exposure leads to extensive apoptosis in vascular smooth muscle cells,[5] human coronary cells,[6] and human umbilical venous endothelial cells (HUVECs).[7,8] It has been further shown that the p38 mitogen-activated protein kinase (MAPK) is a downstream effector of the oxLDL-induced apoptotic process.[5] Little is known, however, about the upstream molecular mechanisms that regulate this process.

In our efforts to identify novel signal-transduction pathways further upstream, we came across integrin-linked kinase (ILK) by virtue of its upregulation in oxLDL-exposed HUVECs. ILK is a novel, ankyrin repeat–containing serine-threonine kinase that exhibits cell type–dependent activation and inhibition.[9] ILK is expressed in cardiac and skeletal muscles.[10] It has been demonstrated that overexpression of ILK in epithelial cells results in the stimulation of anchorage-independent cell growth and cell-cycle progression. In addition, ILK has been shown to be overexpressed in a number of malignancies[10] and is implicated in tumorigenesis.[11] In epithelial and tumor cells, ILK expression suppresses apoptosis.[12,13] The function of this gene in the cardiovascular system, however, is unknown.

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In the present study, we show that ILK is induced transcriptionally and translationally in endothelial cells exposed to oxLDL. We also show that ILK can protect vascular endothelial cells from apoptosis induced by oxLDL. Furthermore, oxLDL-induced p38 MAPK activation and caspase activation were inhibited significantly by ILK expression. Finally, oxLDL-induced phosphorylation of bcl-2 and down-regulation of bcl-xl also were inhibited by ILK overexpression.

Methods

Cell Culture

SVEC4-10 is a mouse lymphoid vein endothelial cell line transformed by simian virus 40 (American Type Culture Collection, Manassas, Va). It was cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). The HUVECs were obtained from Clonetics Human Cell Systems (San Diego, Calif) and were cultured in endothelial cell growth medium (EGM-MV) according to manufacturer instructions.

Preparation of oxLDL

Native human LDLs were purchased from Calbiochem. oxLDL was prepared according to a published protocol.[5] Obtained oxLDL was extensively oxidized and moved 2 to 3 times faster on agarose gel electrophoresis than did the native LDL.

Plasmid Construction and Adenovirus Production

The AdEasy adenovirus-packaging system, including plasmid pAdtrack-cytomegalovirus (CMV), pAdeasy-1, and *E coli BJ5183* (a recombination-competent strain) cells, was kindly provided by Drs T.-C. He and B. Vogelstein[14] (The Johns Hopkins Medical School, Baltimore, Md). The human ILK gene was amplified by reverse-transcriptase polymerase chain reaction (PCR) from total RNA extracted from the Fadu cells (a human squamous cell carcinoma cell line). The forward primer sequence was 5′-GCTCGAGACTATGGACGACATTTTCACTCAG-3′, and the reverse primer was 5′-GGATATCCTAAGCATAATCTGGAACATCATATGGATACTTGTCCTGCATCTTCTC-3′. They were designed according to gene bank sequence HSU40282. XhoI and EcoRV sites (underlined) were engineered into the forward and reverse primers, respectively. A 9-amino acid influenza hemagglutinin (HA) epitope tag sequence was fused to the 3 termini of ILK sequence for Western blot detection. The PCR product was cloned by TA cloning (Invitrogen, Carlsbad, Calif) and sequence verified. The cloned human ILK gene was then subcloned into the XhoI/EcoRV sites of the pAdtrack-CMV vector to produce the pAdtrack-CMV/hILK plasmid. Packaging and production of a recombinant adenovirus carrying the human ILK gene was achieved using the AdEasy system according to published protocols.[14]

Northern and Western Blot Analysis

Northern and Western blot analyses were carried out according to established protocols.[15] For Northern blots, the subcloned human ILK (hILK) fragment was used as the probe. An antibody to the HA tag (Roche Molecular Biology) was used to detect recombinant hILK expression in the Western blot analysis of some samples. Another anti-ILK antibody (Upstate Biotechnology) also was used to detect ILK protein expression. An anti–β-actin antibody was used as a loading control (NeoMarkers Corp). Anti-caspase 3 and anti-caspase 9 antibodies were from New England BioLabs. Anti-bcl2 and anti–bcl-xl antibodies were purchased from Pharmingen.

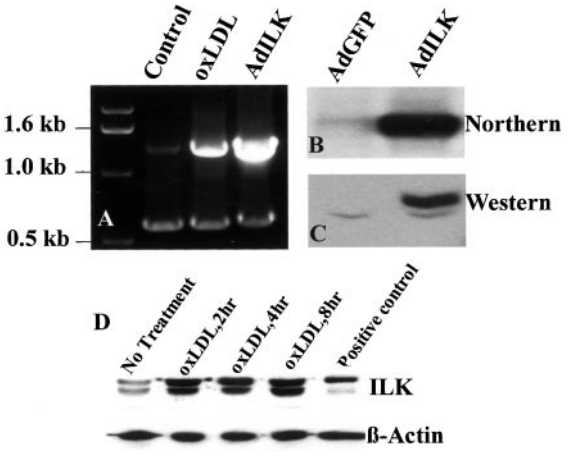

Figure 1. ILK gene expression analysis in HUVECs and SVECs. A, Reverse-transcriptase PCR analysis of ILK gene expression in HUVECs. Total RNA from control HUVECs, HUVECs treated with oxLDL (300 μg/mL for 36 hours), and HUVECs infected with AdILK were isolated and reverse-transcribed. PCR amplification was carried out using 2 pairs of primers in the same reaction: one that amplified a 1.36-kb human ILK cDNA fragment and one that amplified a 600-bp control GAPDH fragment. Note the difference in the level of the 1.36-kb fragment versus the similarity in the 0.6-kb fragment. B, Northern blot analysis of AdILK- and AdGFP-infected SVECs. SVECs at 70% confluence were infected with AdILK and AdGFP, respectively, at a multiplicity of infection (MOI) of 20. After 24 h, total RNA was isolated from the infected SVECs and a Northern blot analysis was carried out. A PCR-amplified ILK fragment (see Methods for details) was used as the probe. C, Western blot analyses of AdILK- and AdGFP-infected SVECs. SVECs at 70% confluence were infected with AdILK and AdGFP, respectively, at an MOI of 20. After 24 h, total protein was isolated from the infected SVECs, and Western blot analysis was carried out. An antibody against the HA tag, which is present in the adenovirus-carried ILK gene (see Methods for more details), was used to detect the presence of the enforced expression of the ILK protein. D, Western blot analysis of the oxLDL-induced ILK protein levels in HUVECs. HUVECs at 70% confluence were treated with oxLDL (300 μg/mL), and total cellular protein was extracted and analyzed by use of an anti-ILK antibody. A total of 4 ILK bands were detected. Shown are 2 major bands at 50 and 59 kDa. β-actin was used as a loading control. The rightmost lane was control cell lysate obtained from Upstate Biotechnology.

Cell Death Assays

Cell death in oxLDL-treated SVECs and HUVECs was evaluated by 2 independent methods: The first was the Hoechst33342 staining of nuclear DNA according to established protocols. The second was the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which was carried out according to an established protocol.[16]

Phospho-p38 MAPK Assay

p38 MAPK activity was measured using a nonradioactive immobilized phospho-p38 MAPK assay kit purchased from Cell Signaling Technology, Inc. Manufacturer's instructions were followed to carry out the assay.

Results

Reverse-transcription PCR was used to characterize the expression level of the ILK gene in HUVECs that had been treated with oxLDL. As shown in Figure 1A, the ILK gene is clearly overexpressed in oxLDL-treated HUVECs. The data also were confirmed by Western blot analysis (Figure

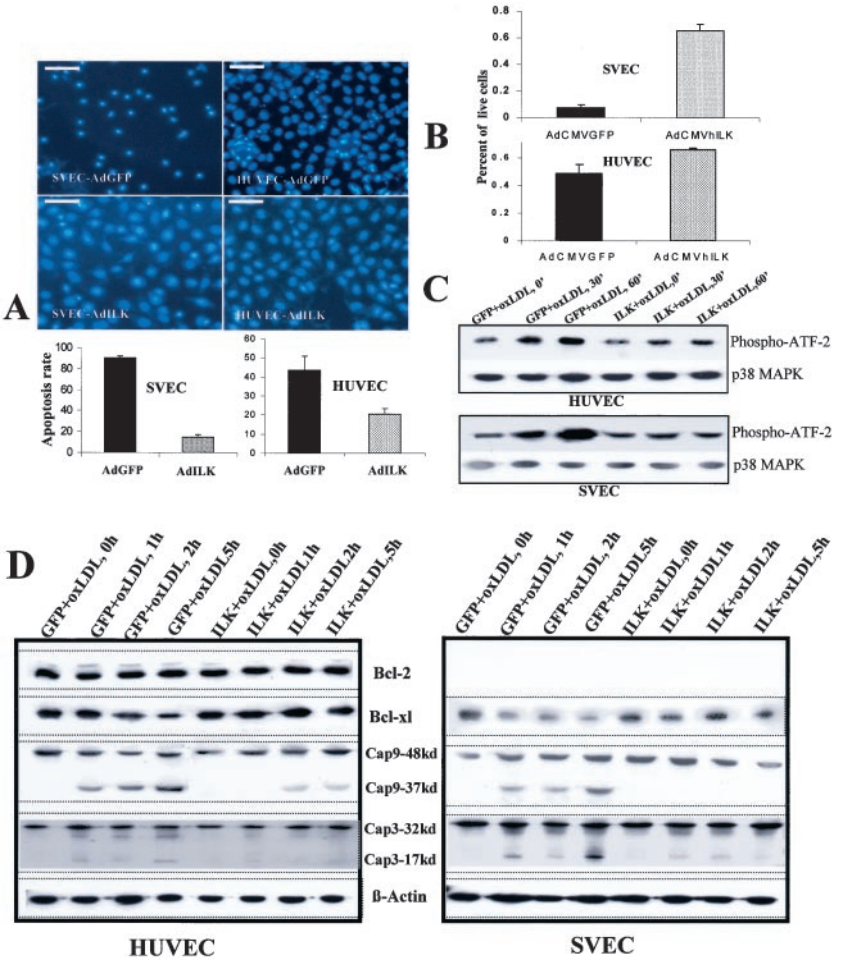

Figure 2. Analysis of oxLDL-induced apoptosis and ILK expression. A, Evaluation of oxLDL-induced apoptosis by use of the Hoechst33342 staining method. SVECs and HUVECs were grown to 70% to 80% confluence and were subsequently infected with AdGFP and AdILK, respectively. After 12 to 18 h, the cells were treated with oxLDL. At 24 h (for SVECs) and 36 h (for HUVECs) after oxLDL treatment, the cells were fixed in 100% methanol, stained with Hoechst 33342, and evaluated for apoptosis. Top panels show fluorescence photomicrographs of cells treated with oxLDL and stained with Hoechst33342. Bars=50 μm. Lower panels show the percentage of apoptosis in each treated cell population. About 1000 cells were counted for each data point and 5 independent experiments were carried out. B, Evaluation of oxLDL-induced apoptosis using the MTT method. SVECs and HUVECs were grown to 70% to 80% confluence and subsequently were infected with AdGFP and AdILK, respectively. After 12 to 18 h, the cells were treated with oxLDL. At 24 h (for SVECs) and 36 h (for HUVECs) after oxLDL treatment, the cells were subjected to MTT analysis. Note that the optical density (OD at 550 nm) value correlates linearly with the integrity of the mitochondria and therefore with cell survival. C, The kinase activity of p38 MAPK in oxLDL-treated SVECs and HUVECs. Shown is the Western blot analysis of the amount of phospho-ATF-2 (Thr71), which is detected by use of an antibody that selectively binds phosphorylated ATF-2 protein (a specific substrate of p38 MAPK). The results were obtained after unphosphorylated ATF-2 was incubated with immunoprecipitated active p38 using an immobilized monoclonal phospho-specific antibody to p38 MAPK (Thr180/Tyr182). Top panel shows results obtained with HUVECs that had been infected with AdGFP (lanes 1 to 3) and AdILK (lanes 4 to 6) and subsequently treated with oxLDL(300 μg/mL). Bottom panel shows results obtained with SVECs that had been infected with AdGFP (lanes 1 to 3) and AdILK (lanes 4 to 6) and subsequently treated with oxLDL (25 μg/mL). Western blot analysis using an antibody against the total p38 protein was used as protein loading control. D, Examination of effector proteins in the apoptotic machinery. HUVECs or SVECs at 70% confluence were infected with AdGFP (control vector) and AdILK for 24 hours at multiplicity of infection of 20. The cells were then treated with oxLDL for duration at different concentrations (25 μg/mL for SVECs and 300 μg/mL for HUVECs). For bcl-2, the top light band is the phosphorylated form of the protein. For caspase 9 (Cap9), the 48-kDa band is the intact form and the 37-kDa band is the cleaved form. For caspase 3 (Cap3), the 32-kDa band is the full-length form and the 17-kDa form is the cleaved form. Note the difference (for the cleaved forms) between the AdGFP-infected cells versus AdILK-infected cells in both SVECs and HUVECs.

1D). It is clear that ILK expression was upregulated beginning at 2 hours and lasting until ≥8 hours after oxLDL exposure.

In order to evaluate the function of ILK, an adenovirus encoding the human ILK gene under the control of a CMV promoter was engineered. The constructed virus, AdILK, mediated the efficient expression of ILK gene in both the HUVECs (Figure 1A) and the SVECs (Figure 1B and 1C). The artificially enforced expression of the human ILK gene affected neither the growth rate nor the morphology of AdILK-infected cells (data not shown).

Exposure of endothelial cells to oxLDL has been shown to induce apoptosis in a time- and concentration- dependent manner. Two assays were used to evaluate the effects of ILK expression on oxLDL-induced cell death. In the first method, Hoechst33342 was used to stain the nuclei of oxLDL-treated cells. Apoptotic cells typically are identified as those cells that possess significantly smaller, condensed, and fragmented nuclei under a fluorescence microscope. Exposure of SVECs to oxLDL (at 25 μg/mL for 24 hours) caused a significant amount of apoptosis. Adenovirus-mediated expression of a reporter gene, the green fluorescence protein (GFP), had no effect on oxLDL-mediated cell death (Figure 2A). Cell death was observed in ≈90.2±1.5% of cells. AdILK-infected SVECs, however, showed a significant reduction in oxLDL-mediated cell death (Figure 2A), with only 14.8±2.2% of the cells showing apparent apoptosis.

Similar observations were made in HUVECs, except for the fact that HUVECs were more resistant to the cell-killing effects of oxLDL (300 μg/mL for 36 hours caused significant apoptosis). Adenovirus-mediated ILK expression signifi-

cantly protected HUVECs from oxLDL-induced apoptosis (Figure 2A), with only 20.5±2.8% of the cells demonstrating apoptosis in the AdILK-infected group versus 43.4±7.3% in the AdGFP-infected control group.

The protective effects of artificially expressed ILK gene also were confirmed by use of a second approach for evaluating cellular death: the MTT assay. Instead of measuring apoptotic cells, the MTT assay allows one to quantify the amount of live cells by detecting active enzymes in the mitochondria. It is based on the conversion of the yellow tetrazolium salt MTT to the blue formazan derivative by mitochondria enzymes in viable cells.[16] We used an improved version of the assay.[17] Our MTT assays confirmed the results of the Hoechst33342 staining. In both the HUVECs and the SVECs, AdILK infection significantly protected the cells from oxLDL-induced cytotoxicity (Figure 2B). In AdGFP-treated HUVECs, oxLDL treatment left 59.5% of the cells to survive, whereas AdILK treatment left 80.3% of the cells to survive (Figure 2B). In SVECs, AdGFP infection left 10.4% of the cells to survive after oxLDL treatment, whereas AdILK infection left 82.2% of the cells to survive (Figure 2B).

At the molecular level, oxLDL-induced apoptosis in endothelial cells has been associated with the activation of the p38 stress-activated protein kinase (MAPK).[5,18] Indeed, this is confirmed in both the HUVECs and the SVECs exposed to apoptosis-causing doses of oxLDL (Figure 2C). When SVECs and HUVECs were treated with oxLDL, there was a significant upregulation of p38 MAPK activity. Adenovirus-mediated GFP expression had no effect on this upregulation, whereas adenovirus-mediated ILK expression suppressed it. These data indicate that the antiapoptotic effect of ILK functions upstream of the p38 MAPK gene.

Because the protein kinase B (PKB/Akt) signal-transduction pathway has been implicated as a downstream molecule of the ILK gene, the total protein levels of PKB/akt as well as the phosphorylated forms of the protein were analyzed by Western blot in both HUVECs and SVECs. Results indicate that both HUVECs and SVECs expressed low but detectable amounts of PKB/akt, but no phosphorylated forms of the protein could be detected either before or after oxLDL treatment (data not shown). This may reflect a cell-type variation in the functions of PKB/akt.

Additional experiments were also conducted to examine the status of several effector genes involved in the apoptotic signal-transduction pathway. The expression of bcl2 was examined in the HUVECs. The level of the bcl2 protein showed no obvious changes. However, the phosphorylation patterns of bcl2 were different between the control GFP expression and ILK expression groups. In the AdGFP-infected group, oxLDL treatment caused a clear increase in the level of phosphorylated bcl2 (top light band, Figure 2D). On the other hand, in the AdILK-infected group, oxLDL treatment caused no changes in the levels of the phosphorylated bcl2. Examination of bcl-xl in both SVECs and HUVECs demonstrated differences in the overall levels of the protein. In the control AdGFP group, oxLDL caused a clear reduction in bcl-xl levels, whereas no changes were observed in the AdILK group. These results demonstrate the involvement of bcl2 family of proteins in oxLDL-induced endothelial cell death and a role for ILK in regulating their functions.

When caspase 9 was analyzed, cleavage of the full-length protein was clear in the oxLDL-treated, AdGFP-infected cells and was significantly reduced (HUVECs) or nearly absent (SVECs) in the oxLDL-treated, AdILK-infected cells (Figure 2D). A similar pattern was observed for caspase 3 in SVECs: Clear cleavage was induced in oxLDL-treated, AdGFP-infected cells but was significantly reduced in oxLDL-treated, AdILK-infected cells.

Conclusions

Our studies indicate that oxLDL exposure induced the expression of the ILK gene. We further show that the preexpression of the ILK gene can substantially prevent oxLDL-induced apoptosis. Inasmuch as the ILK gene is expressed abundantly in cardiac and skeletal muscles and has been shown to be an important regulator of cell–extracellular matrix interactions that lead to cell survival, our results strongly suggest that ILK is an important player in oxLDL-induced endothelial cell apoptosis. It is also notable that the manner in which ILK is induced by stress induction resembles that of the heat shock proteins. This suggests that ILK is part of the signal-transduction feedback loop in endothelial cells that is activated in the event of cellular stress. The suppression of p38 MAPK activity and other effectors by ILK in cellular apoptotic machinery further points to potential molecular pathways through which ILK may participate in regulating apoptosis in endothelial cells and in oxLDL-induced atherosclerogenesis.

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References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
2. Steinberg D. Lewis A. Conner Memorial Lecture: oxidative modification of LDL and atherogenesis. *Circulation*. 1997;95:1062–1071.
3. Okura Y, Brink M, Itabe H, et al. Oxidized low-density lipoprotein is associated with apoptosis of vascular smooth muscle cells in human atherosclerotic plaques. *Circulation*. 2000;102:2680–2686.
4. Thomas MJ. Physiological aspects of low-density lipoprotein oxidation. *Curr Opin Lipidol*. 2000;11:297–301.
5. Jing Q, Xin SM, Cheng ZJ, et al. Activation of p38 mitogen-activated protein kinase by oxidized LDL in vascular smooth muscle cells: mediation via pertussis toxin-sensitive G proteins and association with oxidized LDL-induced cytotoxicity. *Circ Res*. 1999;84:831–839.
6. Napoli C, Quehenberger O, De Nigris F, et al. Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. *Faseb J*. 2000;14:1996–2007.
7. Walter DH, Haendeler J, Galle J, et al. Cyclosporin A inhibits apoptosis of human endothelial cells by preventing release of cytochrome C from mitochondria. *Circulation*. 1998;98:1153–1157.

8. Claise C, Edeas M, Chaouchi N, et al. Oxidized-LDL induce apoptosis in HUVEC but not in the endothelial cell line EA.hy 926. *Atherosclerosis*. 1999;147:95–104.
9. Novak A, Hsu SC, Leung-Hagesteijn C, et al. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc Natl Acad Sci U S A*. 1998;95:4374–4379.
10. Chung DH, Lee JI, Kook MC, et al. ILK (beta1-integrin-linked protein kinase): a novel immunohistochemical marker for Ewing's sarcoma and primitive neuroectodermal tumour. *Virchows Arch*. 1998;433: 113–117.
11. Wu C, Keightley SY, Leung-Hagesteijn C, et al. Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem*. 1998;273:528–536.
12. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene*. 2000;19:3811–3815.
13. Persad S, Attwell S, Gray V, et al. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc Natl Acad Sci U S A*. 2000;97:3207–3212.
14. He TC, Zhou S, da Costa LT, et al. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A*. 1998;95: 2509–2514.
15. Ausubel F, Brent R, Kingston P, et al, eds. *Current Protocols in Molecular Biology*. 2nd ed. New York: Wiley; 1994:4.5, 10.9.
16. Garn H, Krause H, Enzmann V, et al. An improved MTT assay using the electron-coupling agent menadione. *J Immunol Methods*. 1994;168: 253–256.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.
18. Kusuhara M, Chait A, Cader A, et al. Oxidized LDL stimulates mitogen-activated protein kinases in smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol*. 1997;17:141–148.

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