Adenovirus-Mediated Overexpression of Dominant-Negative Mutant of c-Jun Prevents Intercellular Adhesion Molecule-1 Induction by LDL

A Critical Role for Activator Protein-1 in Endothelial Activation

Nanping Wang, Lynne Verna, Hai-ling Liao, Alex Ballard, Yi Zhu, Michael B. Stemerman

Abstract—Low density lipoprotein (LDL) induces intercellular adhesion molecule-1 (ICAM-1) gene expression and leads to endothelial cell (EC) leukocyte adhesion. However, the transcriptional mechanism for LDL-induced EC perturbation remains to be fully explained. Activator protein-1 (AP-1) is induced after the exposure of ECs to LDL. In the present study, a regulated adenovirus expressing a dominant-negative mutant of c-Jun (TAM-67) was used to examine the role of AP-1 in the LDL-induced ICAM-1 activation. Overexpression of TAM-67 specifically inhibited AP-1 activation and prevented the LDL-activated surface expression of ICAM-1 protein in human umbilical vein ECs and human coronary artery ECs. Northern analyses and promoter transactivation assays indicated that this effect of TAM-67 was likely mediated through a suppression of the transcriptional regulation of the ICAM-1 gene. Functionally, TAM-67 attenuated leukocyte adherence to ECs in response to LDL. Furthermore, electrophoresis mobility shift assays and site-directed mutagenesis suggested that an AP-1–like motif in the promoter region of the human ICAM-1 gene was a critical cis element for LDL induction. These results, for the first time, provide evidence suggesting that AP-1 is a major regulatory mechanism leading to endothelial activation. (Arterioscler Thromb Vasc Biol. 2001;21:1414-1420.)

Key Words: LDL ■ endothelium ■ adhesion molecules ■ transcription factors ■ gene expression

Low density lipoprotein is among the major risk factors and predictors for the development of atherosclerosis. LDL perturbs endothelial function, which is manifested as an increase in free radicals, a decrease in NO production, an increase in plasminogen activator inhibitor-1, and recruitment of monocytes via the induction of vascular adhesion molecules. However, unlike agents such as inflammatory cytokines and endotoxins, native LDL does not appear to be a stimulus of nuclear factor-κB (NF-κB), a transcription factor that is thought to play a predominant role in endothelial cell (EC) activation. Rather, LDL induces other transcription factors, such as Egr-1 and activator protein-1 (AP-1). In particular, LDL provokes a sustained elevation of c-Jun expression, AP-1 DNA binding, and transactivation activity in ECs. Moreover, the LDL induction of AP-1 is preceded by a rapid activation of c-Jun N-terminal kinase (JNK), the immediate upstream activator for c-Jun. Recent observation in hypercholesterolemic rabbits revealed a persistent activation of JNK in atherosclerotic intima, indicating a possible link between AP-1 activation and atherosclerosis.

Exposure to LDL increases endothelial expression of intercellular adhesion molecule-1 (ICAM-1), which is a counterreceptor for leukocyte function–associated antigen-1 and is a major component of inflammatory cell recruitment to ECs. Although extensive evidence has pointed to an important role of ICAM-1 in atherogenesis, the transcriptional mechanism by which LDL regulates this adhesion molecule has been largely unknown. Thus, the aim of the present study was to examine whether AP-1 is functionally important in ICAM-1 induction and the ensuing EC phenotypic activation in response to LDL. By using a tightly regulated adenovirus expressing a dominant-negative mutant of c-Jun, we have demonstrated that specific inhibition of AP-1 potently attenuated LDL-induced ICAM-1 expression and monocyte recruitment. Furthermore, an AP-1–like motif in the promoter region of the human ICAM-1 gene was identified as a critical cis element for LDL induction. These results, for the first time, provide evidence suggesting that AP-1 is a major regulatory mechanism leading to endothelial activation.

Methods

Cell Culture and LDL

Human umbilical vein ECs (HUVECs) were isolated from umbilical cords and maintained in EC medium containing 20% FBS, 20 mmol/L HEPES (pH 7.4), 5 ng/mL recombinant human fibroblast growth factor, 90 μg/mL heparin, and antibiotics. Human coronary...
artery ECs (HCAECs) were purchased from Clonetics, cultured in EC growth medium (EGM-2 MV, Clonetics) with 5% FBS, and changed to EC medium after confluence. THP-1, a human monocyte cell line (American Type Culture Collection), was grown in RPMI 1640 containing 10% FBS. LDL (1.019 g/mL; density <1.063 g/mL) was isolated from human plasma by a 2-step ultracentrifuge method as described previously. To generate adenoviruses expressing the dominant-negative c-Jun mutant (AdTAM-67), the cDNA fragment encoding a truncated (residues 3 to 122) c-Jun (TAM-67, provided by M.J. Birrer, NCI, Rockville, MD) was subcloned into pAdlox and recombined with an E1- and E3-deleted adenovirus-5 genome DNA (gifts from Barry Forman, City of Hope, Duarte, Calif). The plasmids were cotransfected to normalize transfection efficiency. Bar14:5 The sequences of oligonucleotides are as follows: (1) ICAM–AP-1b (92–1290), 5'-GCT GCC TCA GTT TCC-3'. (2) ICAM–AP-1a (321), 5'-TAG ACC GTG ATT CAA CAG-3'. (3) ICAM–AP-1c (321), 5'-TAG ACC GTG ATT CAA GCT TAG-3'. (4) ICAM–NF-κB1 (536), 5'-GCC CGG GGA TTC CTG GGC CC-3'. (5) ICAM–NF-κB2 (223), 5'-TTT AGC TTG GAA ATT CCG GAG CT-3'.

Electrophoretic Mobility Shift Assay
An electrophoretic mobility shift assay was performed as described previously.14 The sequences of oligonucleotides are as follows: (1) ICAM–AP-1a (1290), 5'-TGG CCA GTG ACT GCG AGC CCC-3'; (2) ICAM–AP-1b (940), 5'-GCT GCT GCC TCA GTT TCC CAG-3'; (3) ICAM–AP-1c (321), 5'-TAG ACC GTG ATT CAA GCT TAG-3'; (4) ICAM–NF-κB1 (536), 5'-GCC CGG GGA TTC CTG GGC CC-3'; and (5) ICAM–NF-κB2 (223), 5'-TTT AGC TTG GAA ATT CCG GAG CT-3'.

Plasmids and Transfection
The ICAM-1 promoters/reporters were constructed by subcloning progressively truncated segments of a 1344-bp 5' flanking region of the human ICAM-1 gene (provided by Dr T. Parks, Boehringer-Ingelheim Pharmaceuticals Inc, Ridgefield, Conn) into pGL3-basic (Promega). The 7 × AP-1-Luc and 5 × NF-κB-Luc are luciferase reporters containing 7 × AP-1 binding sites or 5 × κB sites (Stratagene). Plasmid expressing the yeast transcription factor Gal4 and 4 × UAS-TK-Luc have been previously reported (gifts from Barry Forman, City of Hope, Duarte, Calif).21 The plasmids were transfected into HUVECs by using a cationic lipid-based reagent (Targefect, Targeting System). Cell lysates were harvested to measure luciferase activity. In all transfection experiments, an RSV-β-gal plasmid was cotransfected to normalize transfection efficiency.

Site-Directed Mutagenesis
Site-directed mutagenesis was performed by using the Quickchange kit (Strategene) according to the manufacturer’s protocol. The mutagenic primers were synthesized to introduce mutations (lowercase letters) into the AP-1-like motif (underlined). The sequence of the primer (forward) is 5'-GCC GTG TAG ACC GTG gTG CgA GCT TAG CCT GGC CC-3' (the reverse primer was complementary to the forward primer). The mutated primers bearing the desired mutations were screened by restriction digestion (the mutation introduced an additional HindIII site) and confirmed by DNA sequencing.

Northern Blot Analysis
Total RNA was isolated by using Trizol reagent (GIBCO-BRL), fractionated, transferred to a nylon membrane, and hybridized to random-primed cDNA probes for the human ICAM-1 and the GAPDH genes.17

Western Blot Analysis
Protein samples were resolved on SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and reacted with rabbit polyclonal antibodies to c-Jun (sc-44, Santa Cruz) and a horseradish peroxidase–conjugated secondary antibody (sheep anti-rabbit, 1:5000, Sigma Chemical Co) followed by detection by enhanced chemiluminescence (ECL, Amersham).17

Immunofluorescence Staining
After fixation with 4% paraformaldehyde, HUVECs were permeabilized with 1% Triton X-100 and incubated with primary rabbit antiserum for 1 hour. The cells were washed with PBS and then reacted with a fluorescein isothiocyanate–conjugated anti-rabbit antibody. Background fluorescence was determined by using the secondary antibody alone and subtracted. The results were observed with a fluorescence microscope.

Fluorescence-Linked Immunoassay of ICAM-1
HUVECs or HCAECs grown in 6-well plates were reacted with a mouse monoclonal antibody against ICAM-1 (Seratec). The secondary antibody was a fluorescein isothiocyanate–conjugated rabbit anti-mouse IgG. The fluorescence was measured with a fluorescence concentration analyzer (Pandex, IDEXX).9

Monocyte Adhesion Assay
HUVECs were grown to confluence on slides in a chamber (Lab-Tek) and incubated for 4 days with or without LDL. THP-1 cells were infected with AdGFP and AdtTA for 24 hours and then coincubated with the HUVECs for 30 minutes. The nonadhering cells were washed off, and the adhered THP-1 cells were observed and counted by use of a fluorescence microscope.

Statistical Analysis
Quantitative data were expressed as mean±SEM. Statistical analysis was performed with the Student t test. Differences were considered significant at P<0.05.17

Results
Regulated Expression of Dominant-Negative c-Jun in ECs
To directly assess the potential requirement for AP-1 in LDL-triggered EC signaling, we overexpressed a dominant-negative mutant of c-Jun, TAM-67, in ECs. Confluent HUVECs were coinfected with AdTAM-67 and AdtTA. At different time points after infection, cellular proteins were isolated and examined for TAM-67 expression by Western analysis with use of an antibody raised against the conserved DNA-binding domain of c-Jun (SC-44). As shown in Figure 1A, a truncated c-Jun protein (∼30 kDa) was readily detected 24 hours after infection. To regulate the TAM-67 expression, adenovirally infected ECs were maintained in medium containing varying concentrations of tetracycline (Tc, 0–1.0 µg/ml). Tc arrested the TAM-67 expression in a dose-dependent fashion. Endogenous wild-type c-Jun protein was also detected at a low level under basal conditions and was not suppressed by TAM-67. However, overexpression of TAM-67 appeared to decrease the induction of endogenous c-Jun by phorbol 12-myristate 13-acetate (PMA) and, to lesser extent, by LDL. Furthermore, immunofluorescence staining showed that after infection by AdTAM-67 and AdtTA, the mutant was detectable exclusively in the nuclei of ECs (Figure 1B) when stained with the c-Jun antibody SC-44 but not with the c-Jun antibody SC-45, which recognizes the N-terminal epitope (data not shown). In noninfected cells or the infected cells maintained in Tc medium, only the endogenous c-Jun was detected.

Inhibition of AP-1 by Overexpression of TAM-67 in ECs
Transient transfection assays with the AP-1–dependent luciferase reporter plasmid, 7 × AP-1-Luc, were carried out to determine the functional efficacy of TAM-67 for suppressing...
AP-1 activity in endothelial cells. HUVECs were infected with AdTAM-67 plus AdtTA and then transfected with 7 × AP-1-Luc (Figure 1C). The transfected ECs were then treated with LDL for 48 hours and measured for luciferase activity. The results showed that TAM-67 suppressed LDL-induced AP-1 activation. Furthermore, luciferase reporters for NF-κB (5 × NF-κB-Luc) and for the yeast transcription factor Gal4 (4 × UAS-TK-Luc) were used to evaluate the effect of the dominant-negative c-Jun on other specific or general transcriptional mechanisms. As shown in Figure 1C, the NF-κB and the Gal4-dependent reporters were activated by exposure to PMA and overexpression of Gal4, respectively. Basal activity and induced activity of these transcription factors were unaffected by TAM-67 at the same level at which it significantly suppresses AP-1 activity.

Blocking AP-1 Inhibited LDL Induction of ICAM-1 in ECs

To examine whether inhibition of AP-1 could prevent ICAM-1 induction by LDL, confluent HUVECs were infected with AdTAM-67 and AdtTA and, thereafter, maintained in medium (containing 20% FBS) with or without Tc. After infection, the cells were exposed to LDL for 4 days. Total RNA was isolated and hybridized for ICAM-1 transcripts. As shown in Figure 2A, the ICAM-1 mRNA expression induced by LDL was silenced by TAM-67, whereas the expression of GAPDH, a housekeeping gene, was not affected. Subsequently, promoter transactivation assays were performed to examine whether a transcriptional mechanism accounted for the inhibitory effect of TAM-67. As shown in Figure 2B, LDL induction of ICAM-1 promoter activity was markedly inhibited by TAM-67, indicating that TAM-67 decreases LDL-induced ICAM-1 mRNA by suppressing the promoter activation. Next, surface expression of ICAM-1 was examined by immunofluorescence to determine whether the suppressive effect also occurs at the protein level. Interestingly, in HUVECs and HCAECs, the ICAM-1 protein level was increased after exposure to LDL, and the induction was diminished by overexpression of TAM-67 (Figure 2C). Thus, it is suggested that the AP-1 may be an important regulator in LDL-induced ICAM-1 expression.

Inhibition of AP-1 Attenuates LDL-Induced EC Monocyte Adhesion

To examine whether inactivation of AP-1 in ECs functionally modulates EC-monocyte interaction in response to LDL, ECs were coinfected with AdTAM-67 and AdtTA, in the presence or absence of Tc, and exposed to LDL. THP-1 cells were preinfected with AdGFp and AdtTA for 24 hours for direct visualization by fluorescence microscopy. These cells were incubated with LDL-exposed ECs for 30 minutes. As shown in Figure 3, LDL caused an ~3-fold increase in monocyte binding to ECs. Overexpression of TAM-67 had no effect on the basal level of monocyte adherence but significantly decreased the LDL-induced EC-monocyte interaction. Therefore, inhibition of AP-1 activity in ECs could functionally attenuate the proinflammatory effects of LDL.
LDL Induction of Human ICAM-1 Promoter

Structural analysis of the human ICAM-1 gene has revealed multiple AP-1–like and NF-κB–like motifs within a 1.3-kb 5′ flanking region (Figure 4). To delineate the promoter region(s) responsible for LDL induction, we transfected HUVECs with reporters fused to a series of 5′ deletion mutants of the 1.3-kb fragment and exposed the cells to LDL. LDL caused a moderate induction of the 1.3-kb ICAM-1 promoter/reporter. However, deletion of the distal segment (−2130 to −942) decreased the basal activity but increased the promoter responsiveness to LDL. Further deletion of a segment containing an AP-1/Ets repeat (AP-1b) and a consensus NF-κB site (ICAM-κB1) caused little decrease in both basal activation or the LDL responsiveness. The most critical cis element(s) appears to reside in the 168-bp region (−445 to −277). Thus, it is suggested that the proximal AP-1 site (AP-1c) may be a candidate element to mediate the promoter responsiveness to LDL.

Proximal AP-1-Like Motif Is Required for LDL Induction

To examine whether LDL has an effect on the several putative binding motifs for AP-1 and NF-κB transcription factors in the human ICAM-1 promoter, an electrophoretic mobility shift assay was carried out with oligonucleotide containing the sequences corresponding to the specific AP-1 or NF-κB sites (Figure 5). LDL enhanced protein binding to the probe containing the proximal AP-1 sequence (AP-1c), caused moderate binding to the oligomers containing the AP-1/Ets repeats (AP-1b), and caused little binding to the oligomers containing the distal AP-1 (AP-1a). However, LDL had no detectable effect on the nuclear protein binding to

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oligomers for either of the NF-κB sites. Specificity of the DNA binding activity was confirmed by using a 100-fold molar excess of the cold oligonucleotides to compete out the retarded bands (data not shown).

To specifically examine the functional contribution of the proximal AP-1 site to LDL-mediated transcriptional activation of ICAM-1, a mutant construct was generated to disrupt the proximal AP-1 site (−445mut). As shown in Figure 6, site-directed mutagenesis of the proximal AP-1 motif largely abolished the promoter responsiveness to LDL. Thus, the proximal AP-1–like motif appears to be a critical LDL-responsive element in the ICAM-1 promoter.

Discussion

LDL is considered to be the main atherogenic class of lipoproteins, and an elevated level of LDL is one of the most important risk factors for atherosclerosis and cardiovascular morbidity. Increased levels of plasma LDL cholesterol are correlated with increased risk for complications of atherosclerosis, whereas lipid-lowering treatment can reduce coronary events and total mortality. Continuous interaction between plasma LDL and arterial endothelium seems to be one of the factors pertinent to the development of atherosclerosis. Although growing evidence supports the idea that oxidation of LDL may increase its ability to damage endothelial function, there is also an increasing body of evidence showing that a pathophysiological level of nonoxidized native LDL causes endothelial perturbations. In addition to our previous finding that LDL induces the expression of a number of adhesion molecules and increases proinflammatory cell adhesion to HUVECs, recent studies from our laboratory and other groups have largely expanded the ever-growing spectrum of atherogenic effects of native LDL. Together, these observations argue for a pathophysiological role of LDL in the development of atherosclerosis and thrombosis. In search of the molecular mechanisms by which LDL may modulate the endothelial phenotype, we further demonstrate in the present study that (1) LDL-induced ICAM-1 expression and resultant EC-leukocyte adhesion can be attenuated by blocking AP-1 activation with adenovirus-mediated expression of a dominant-negative c-Jun in ECs, and (2) the AP-1–like motif in the ICAM-1 promoter appears to be a major cis element responsible for LDL induction. The present study shows, for the first time, that the AP-1 signaling pathway and its cognate binding motif are of major importance for endothelial activation by LDL.

AP-1 is a family of transcription factors composed of various dimers of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2). Family members form dimers and bind to DNA containing a TPA (12-O-tetradecanoyl-phorbol-13-acetate)-responsive element site to regulate gene expression. AP-1 provides pivotal regulatory roles coupling extracellular signals to key gene-activating events. Deregulation of AP-1 is seen in ECs in response to a variety of pathological stimuli. Particularly, LDL induces a sustained activation of AP-1 in ECs. In contrast to proinflammatory cytokines, LDL lacks an effect on NF-κB. Using adenovirus-mediated overexpression of c-jun and c-fos, we demonstrated that experimental activation of AP-1 is sufficient to cause endothelial activation, independent of NF-κB. The present report further shows that specific inactivation of AP-1 diminished ICAM-1 expression and the ensuing leukocyte recruitment, indicating that LDL, as a chronic vascular agonist, perturbs the EC phenotype through a mechanism that is distinct from an acutely activated pathway.

ICAM-1 is an inducible cell adhesion glycoprotein expressed on the surface of a variety of cell types, including ECs, and plays an important role in leukocyte adhesion and inflammation. It is also implicated in atherosclerosis. Thus, elucidation of the transcriptional control of this molecule is important for understanding the inflammatory response in general as well as the pathogenesis and therapeutic intervention of atherosclerosis. In ECs, ICAM-1 is upregulated in response to a wide variety of stimuli, including proinflammatory cytokines, lipopolysaccharides, PMA, and H₂O₂. These stimuli increase ICAM-1 expression primarily through the activation of ICAM-1 gene transcription. On the other hand, various species of lipoproteins, including LDL, oxidized LDL, Lp(a), and remnant lipoproteins, have been reported to induce the expression of ICAM-1 in ECs. However, to our knowledge, there has been no preceding report identifying the regulatory elements responsible for ICAM-1 induction by these lipoproteins. Analysis of the 1.3-kb human ICAM-1 promoter region revealed multiple binding motifs for various transcription activators, including AP-1, NF-κB, Ets, signal transducers and activators of transcription, and SP-1. Such a complex promoter structure appears to mediate gene induction in response to diverse
AP-1 pathway. To disable all AP-1– containing transcription used in the present study to test the functional role of the region containing this motif did not cause marked reduction of c-Jun. It retains the leucine zipper domain necessary for dimerization with Jun and Fos family members and the N-terminal region responsive to physiological and pathological conditions.

In conclusion, the present results demonstrate that AP-1 activation plays a critical role in endothelial phenotypic perturbation by LDL, which may represent a novel mechanism of endothelial response to LDL cholesterol. It is hoped that this finding may help in understanding the roles of LDL and transcription factors in vascular inflammatory processes and atherogenesis.

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