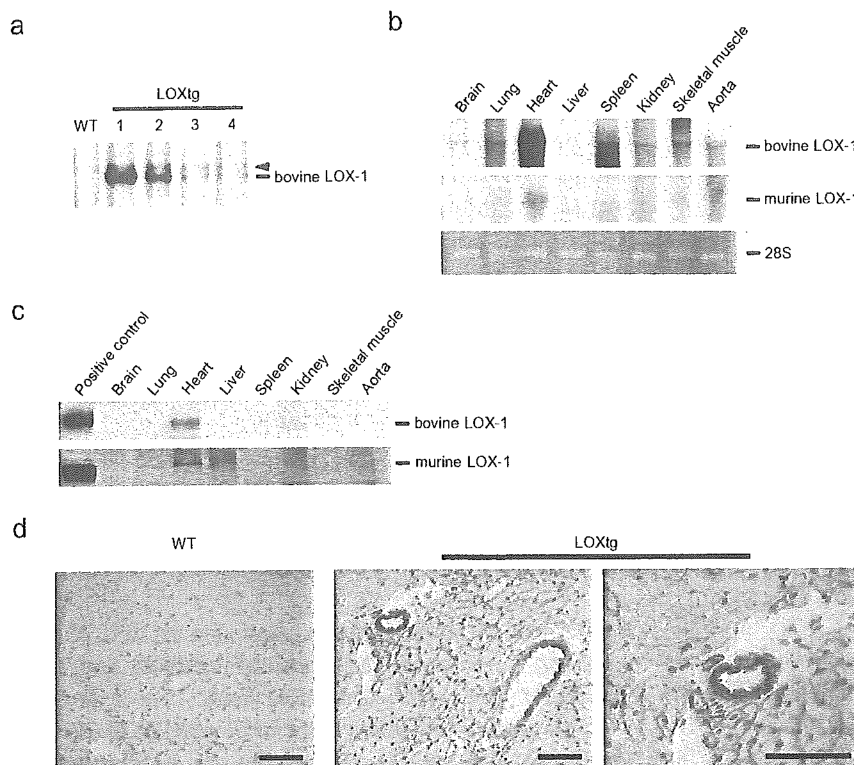

Figure 1. Generation of LOXtg and tissue distribution of transgene expression. a, Southern blot analysis of the transgene. Genomic DNA extracted from mice tail was digested with *EcoRI*, and probed with the radio-labeled fragment of LOX-1 cDNA. Transgene was identified as a single band of 3.2 kb. The arrowhead indicates the endogenous murine preproendothelin-1 promoter with the size of ≈3.5 kb. b, Northern blot analysis. Total RNA from the indicated tissues was loaded and hybridized with the probes of bovine and murine LOX-1. Marked expression of the transgene was observed in the heart. c, Western blot analysis was performed with anti-bovine and anti-murine LOX-1 antibodies. Cells expressing LOX-1 from each species were used as the positive control. A high expression of bovine LOX-1 protein was again observed in the heart. d, Immunohistochemical staining for bovine LOX-1. Frozen sections of heart from WT (left) and LOXtg (center and right) were stained with biotinylated anti-bovine LOX-1 antibody. Scale bars=100 μm.

heart. Assuming that endogenous murine LOX-1 and transgenic bovine LOX-1 have the same signal intensity for an equal molar level of LOX-1, the expression of the transgenic bovine LOX-1 in terms of mRNA level was ≈8-fold greater than that of murine LOX-1 in the heart. Similarly, a marked increase of transgenic bovine LOX-1 protein in the heart was also detected by Western blot analysis. Low levels of the LOX-1 protein were also detected in the kidney (Figure 1c). An analysis of endogenous murine LOX-1 with the same amount of protein revealed a low expression level of endogenous murine LOX-1 in the liver. To identify the cells expressing the transgene in the heart of LOXtg, immunohistochemistry was performed with a monoclonal anti-bovine LOX-1 antibody that does not cross-react with endogenous murine LOX-1 (Figure 1d). Endothelial cells in heart vessels and cardiomyocytes were strongly positive for transgenic bovine LOX-1, whereas heart tissue from wild-type (WT) did not display any positive staining.

Uptake of DiI-OxLDL in Heart

To verify whether the transgenic bovine LOX-1 protein was functional in the heart, OxLDL uptake was analyzed in the cultured tissue of the heart by incubating each slice of the heart tissue with DiI-oxLDL. DiI-OxLDL uptake was enhanced in both endothelial cells and cardiomyocytes in LOXtg, probably reflecting the expression of the LOX-1 transgene in both endothelial cells and cardiomyocytes. The number of DiI-OxLDL–positive vessels was greatly increased in the heart sections from LOXtg compared with those from WT (Figure 2). Inhibition of DiI-OxLDL uptake by simultaneous incubation of an excess amount of unlabeled OxLDL or anti-bovine LOX-1 antibody with DiI-OxLDL further confirmed the specificity of the LOX-1 activity.

Baseline Characteristics of LOXtg

At 16 weeks, there were no significant differences between WT and LOXtg fed a normal chow diet in terms of body weight, mean blood pressure, heart rate, total cholesterol, phospholipid, triglyceride, or NEFA (Table). HPLC analyses further showed that the subfraction profiles of lipoproteins yielded no significant differences in the WT and LOXtg mice.

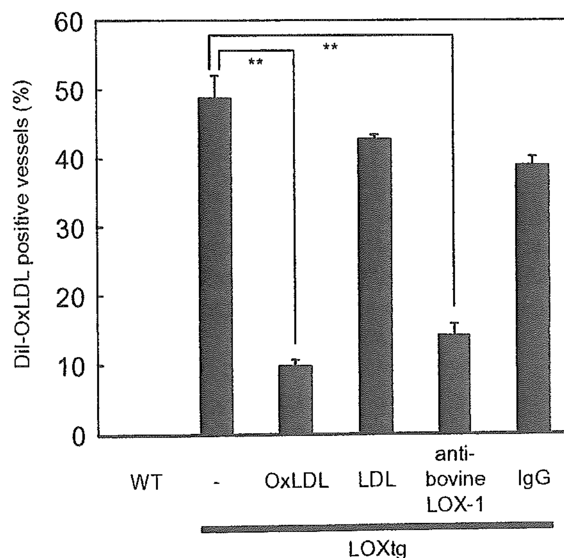

Figure 2. Enhanced OxLDL uptake in heart vessels of LOXtg heart. The WT and LOXtg hearts were sliced and incubated with DiI-OxLDL for 3 hours. The number of DiI-positive vessels was counted in 5 sections from each mouse (n=4). The number of DiI-OxLDL–positive vessels was augmented in the LOXtg heart, and this augmentation was inhibited by the addition of excess OxLDL and anti-bovine LOX-1, but not by LDL or control IgG. $^{**}P<0.01$ compared with DiI-OxLDL without inhibitors.

Hemodynamic and Plasma Lipid Indices of WT (n=14) and LOXtg (n=12) Fed a Normal Chow Diet for 16 Weeks, ApoEKO (n=11) and LOXtg/ApoEKO (n=12) After a 3-Week High-Fat Diet (11 Weeks Old)

	WT	LOXtg	ApoEKO	LOXtg/ ApoEKO
Body wt, g	25.76±0.49	27.66±0.84	19.51±0.88	19.40±0.65
SBP, mm Hg	96.2±2.5	97.7±2.6	93.5±2.1	98.0±2.3
MBP, mm Hg	67.2±2.2	70.6±2.8	64.3±2.1	67.0±1.5
DBP, mm Hg	48.4±2.0	53.9±4.3	49.9±2.4	51.6±1.5
Heart rate, BPM	514.6±16.0	547.3±21.3	526.8±17.4	520.3±18.5
Total cholesterol, mg/dL	77±9	68±6	1030±88	1181±101
Phospholipid, mg/dL	181±11	173±12	440±20	458±20
Triglyceride, mg/dL	114±12	95±13	108±5.0	121±7.9
NEFA, mEq/L	1.67±0.14	1.61±0.17	3.38±0.17	3.40±0.27

Data were collected at 16 weeks of age from animals fed a normal chow diet (WT, LOXtg), and after a 3-week high-fat diet started at 8 weeks of age (apoEKO, LOXtg/apoEKO). SBP indicates systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; NEFA, nonesterified free fatty acid. Values are expressed as the mean±SEM. No significant differences were observed in the above parameters between WT and LOXtg, or apoEKO and LOXtg/apoEKO.

Generation of LOXtg/ApoEKO

To further investigate the effect of LOX-1 overexpression on the heart of LOXtg, LOXtg were cross-bred with apoEKO in a C57BL/6 background. In LOXtg/apoEKO mice, LOX-1 transgene was carried in the same copy number and was expressed at significantly high levels in both endothelial cells in the heart and cardiomyocytes, as in the case with LOXtg (data not shown). We then determined the effect of a high-fat diet on the transgenic mice. ApoEKO and LOXtg/apoEKO were fed a high-fat diet (1.25% cholesterol, 0.5% cholic acid) for 3 weeks starting at the age of 8 weeks. After exposure to the high-fat diet, the total cholesterol, phospholipids, and NEFA exhibited significantly higher levels compared with the levels before the diet. However, no significant differences were observed between apoEKO and LOXtg/apoEKO in terms of body weight, mean blood pressure, heart rate, total cholesterol, phospholipids, triglycerides, NEFA, or subfraction profile of lipoproteins (Table).

Accumulation of OxLDL and the Products of Oxidative Stress

To examine whether the overexpression of LOX-1 accelerates accumulation of OxLDL in the heart under hypercholesterolemia conditions, heart tissue sections were probed with a polyclonal antibody against OxLDL. This antibody does not cross-react with unmodified LDL. In the LOXtg/apoEKO heart, endothelial cells were stained positively, whereas little positive staining was observed in apoEKO (Figure 3a). To determine the oxidative stress in heart overexpressing LOX-1, we measured 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidation product of 2′-deoxyguanosine hydroxylated at the C-8 position. We found that 8-OHdG was detected in endothelial cells of LOXtg/apoEKO, but not in apoEKO under the same conditions (Figure 3b).

Leukocyte Adhesion Molecule Expression and Macrophage Infiltration

To determine the effects of LOX-1 overexpression on the regulation of adhesion molecules and the consequences of this effect, we measured the expression levels of ICAM-1 and VCAM-1 mRNA in the hearts of apoEKO and LOXtg/apoEKO. We found that both genes were expressed at significantly higher levels in the LOXtg/apoEKO (n=7) than apoEKO mice (n=7) (Figure 4a). Immunohistochemical analysis similarly revealed high expression levels of ICAM-1 and VCAM-1 in the endothelial cells of LOXtg/apoEKO. The results are consistent with the mRNA levels of the molecules (Figure 4b and 4c). The expression was not limited to the lesion but also extended to the normal blood vessels in the heart. We further examined the number of macrophages that had infiltrated into the heart tissue. We found that the number of the cells that stained positively with an antimacrophage antibody were increased 6-fold in the LOXtg/apoEKO mice compared with macrophages found in the coronary arteries of the apoEKO mice (LOXtg/apoEKO, 5.60±0.71 cells/mm^2, n=13; apoEKO, 0.88±0.36 cells/mm^2, n=14) (Figure 5). This data suggests that LOX-1 overexpression induces a chronic inflammatory response.

LOX-1 Overexpression Induces Intramyocardial Vasculopathy

To evaluate the physiological consequence of LOX-1 overexpression, we examined the intramyocardial vasculopathy to determine any pathological or histological differences between the 2 male mice lines. The heart tissues were cut into 8-μm thick sections every 40 μm along the long axis from the mitral valve to the aortic valve and stained with Oil red O. LOXtg/apoEKO displayed significant lipid deposition in the blood vessels of the sections (5.8 slices of 10 per mouse) from the coronary artery, whereas apoEKO displayed only occasional deposition in a small number of sections (1.6 slices of 10 per mouse) (Figure 6a). Quantitative analysis of the Oil red O–positive area showed that LOXtg/apoEKO developed an ≈10-fold larger area of lesions than apoEKO (34.31±15.41, n=7; 3.01±1.83, n=8; respectively [$\times 10^{-6}$ Oil red O staining area/heart area]; Figure 6b).

a

apoEKO LOXtg/apoEKO

normal atheroma normal atheroma

OxLDL

non-immune serum

b

apoEKO LOXtg/apoEKO

normal atheroma normal atheroma

8-OHdG

IgG

c

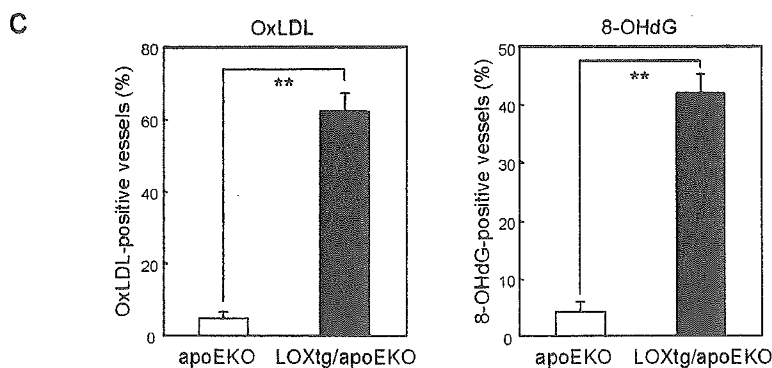

Figure 3. Accumulation of OxLDL and the products of oxidative stress. a, apoEKO and LOXtg/apoEKO heart were immunostained with anti-OxLDL antiserum (upper) and nonimmune serum (negative control; lower). Positive staining was observed in the blood vessels of LOXtg/apoEKO. The right panel shows the staining of the atheroma lesion of LOXtg/apoEKO. Scale bars=100 μm. b, apoEKO and LOXtg/apoEKO heart were immunostained with anti–8-OHdG antiserum (upper) and goat IgG (negative control; lower). A positive signal (arrowheads) was detected again in the blood vessels of LOXtg/apoEKO. Right panel shows the staining of the atheroma lesion of LOXtg/apoEKO. Scale bars=100 μm. c, Percentages of oxLDL- and 8-OHdG positive vessels of the total vessels examined were augmented to a greater extent in the LOXtg/apoEKO (filled bar) than the apoEKO (open bar) heart. Eleven sections from each mouse (n=4) were examined. **P<0.01 compared with apoEKO.

Discussion

Oxidized LDL (OxLDL) is implicated in the pathogenesis of atherosclerosis, including both the formation of foam cells and the induction of endothelial dysfunction. These changes that occur in the vascular wall might be mediated by several receptors for oxidized LDL such as SR-A, CD36, SR-BI, and LOX-1, which together are collectively termed the "scavenger receptor." Macrophages that have migrated into the subendothelial space take up OxLDL, and eventually become lipid-laden foam cells. The role of SR-A and CD36 in this process has been

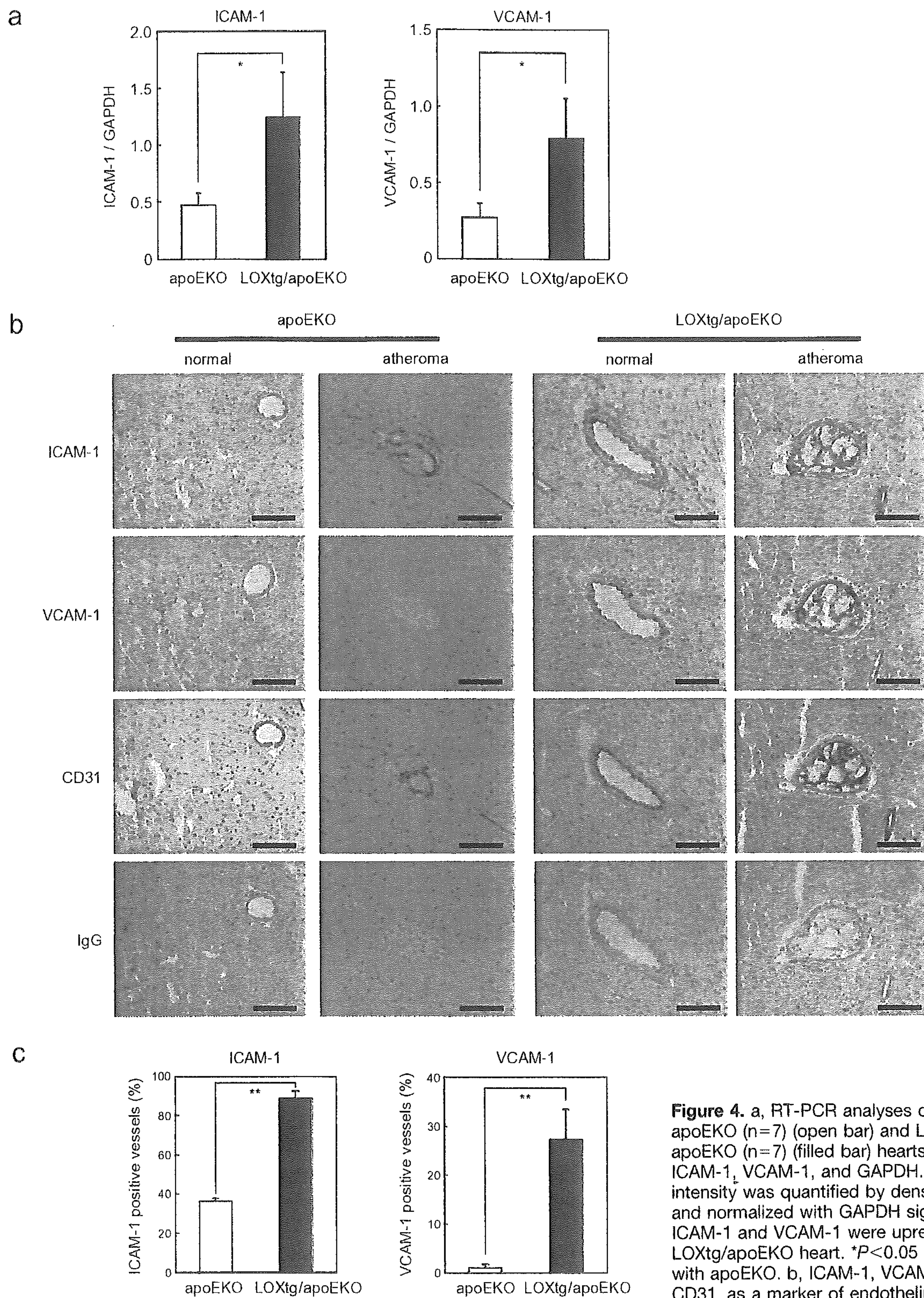

Figure 4. a, RT-PCR analyses of the apoEKO (n=7) (open bar) and LOXtg/apoEKO (n=7) (filled bar) hearts for ICAM-1, VCAM-1, and GAPDH. The band intensity was quantified by densitometry and normalized with GAPDH signal. Both ICAM-1 and VCAM-1 were upregulated in LOXtg/apoEKO heart. **P*<0.05 compared with apoEKO. b, ICAM-1, VCAM-1, and CD31, as a marker of endothelium, were immunohistochemically detected in apoEKO (left) and LOXtg/apoEKO (right). Scale bars=100 μm. Note that the higher expression of VCAM-1 and ICAM-1 is observed in apparently normal vessels. c, The percentages of the ICAM-1 and VCAM-1 stained vessels of the total vessels examined were augmented in the LOXtg/apoEKO (filled bar) compared with the apoEKO (open bar) heart. Eleven sections from each mouse (n=4) were examined. ***P*<0.01 compared with apoEKO.

demonstrated with knockout mice deprived of those genes.[31,32] However, the in vivo function of LOX-1 has yet to be determined.

LOX-1, which is expressed mainly in endothelial cells, has been suggested to initiate endothelial dysfunction. A number of in vitro studies have shown that the generation of superoxide by NADPH oxidase in endothelial cells induced by OxLDL is mediated by LOX-1.[33] Furthermore, in vitro studies indicate that activation of the LOX-1 also initiates a

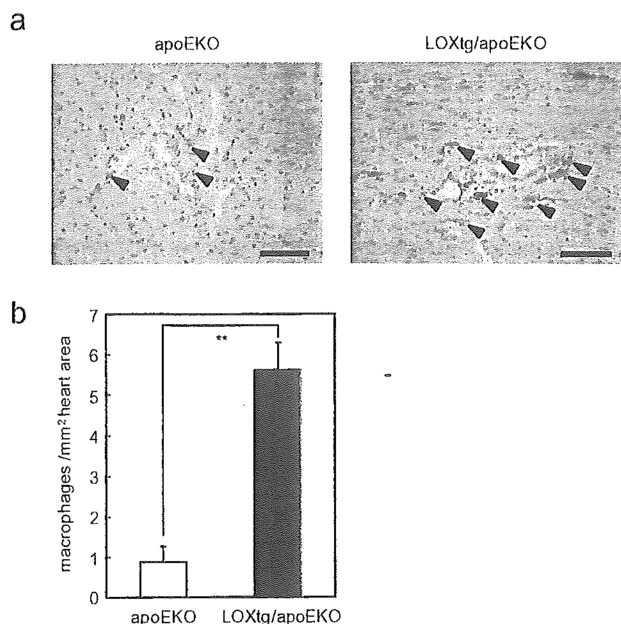

Figure 5. a, Macrophage infiltration (arrowheads) around the coronary artery was immunohistochemically detected with an anti-macrophage antibody. Scale bars=100 μm. b, Number of infiltrated macrophages per heart area was significantly augmented in the LOXtg/apoEKO (n=13) (filled bar) than apoEKO (n=14) (open bar). $^{**}P<0.01$ compared with apoEKO.

reduction in NO release[22] and an upregulation of gene expression, including ET-1, AT1 receptor, MCP-1,[20] and cell-adhesion molecules.[21]

It is clearly demonstrated that in vivo LOX-1 overexpression results in pathological changes of blood vessels under hyperlipidemia. In the transgenic mice, overexpression of the LOX-1 transgene occurs in the blood vessels in the heart and the myocardium at both the mRNA and protein level. In addition, the overexpressed LOX-1 is shown to be functional, because an enhanced uptake of OxLDL in these same tissues was demonstrated. Cross-breeding the LOXtg mice with apoEKO mice established a double transgenic mouse (LOXtg/apoEKO), which was subsequently used to study the pathological consequences of LOX-1 overexpression. Despite the fact that in this mouse model of hyperlipidemia no significant differences were found in the plasma lipoprotein profiles between the LOXtg/apoEKO and apoEKO mice. OxLDL was found to accumulate more in the hearts of LOXtg/apoEKO than apoEKO mice, especially around the coronary blood vessels, where the LOX-1 transgene expression was shown to be particularly high. These results indicate that the pathology associated with the overexpression of LOX-1 is not caused by changes in the amount of VLDL/LDL per se, but rather, to the accumulation of OxLDL in tissues, which accumulation is mediated by the overexpression of functional LOX-1.

There are 3 reasonable interpretations of the present results. First, the modified LDL present in hyperlipidemic plasma may be more effectively bound by the LOX-1 overexpressed on the surface of endothelial cells, which would confirm that LOX-1 is an OxLDL receptor that facilitates OxLDL uptake. Second, LDL that has permeated

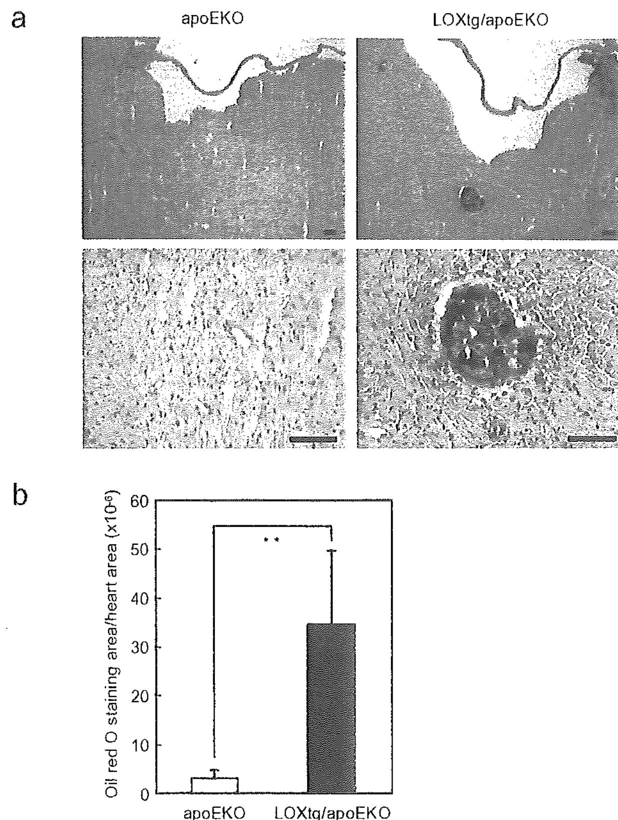

Figure 6. a, Oil red O staining of heart sections of apoEKO and LOXtg/apoEKO fed a 3-week high-fat diet. The coronary arteries of LOXtg/apoEKO were positively stained. Scale bars=100 μm. b, Area stained positively with Oil red O of the hearts from apoEKO (n=8, open bar) and LOXtg/apoEKO (n=9, filled bar). The ratio of the positive staining area was augmented in LOXtg/apoEKO. $^{**}P<0.01$ compared with apoEKO.

through the endothelium and deposited in vascular wall can be oxidized and bound by LOX-1, leading to superoxide generation and increased LDL oxidation. There is evidence that burden of oxidative stress is enhanced by ligands for LOX-1 such as OxLDL[33] and activated platelets.[24] The detection of 8-OHdG, a marker of oxidative stress, in the blood vessels of LOXtg/apoEKO supports the hypothesis that LOX-1 might actually contribute to the increase in oxidative stress under hyperlipidemia. Third, a change in the adhesive capacity of endothelial cells may contribute to the accumulation of OxLDL. Specifically, LOX-1–mediated activation of endothelial cells results in the enhanced expression of chemokines and adhesion molecules.[20,21] The activity of LOX-1 as a leukocyte-adhesion molecule would further enhance the adhesiveness of endothelial cells.[25] These events in turn would further facilitate the infiltration of macrophages into the vascular wall, which infiltrated cells would then scavenge and accumulate OxLDL. In fact, we did show that the expression of ICAM-1 and VCAM-1 is indeed enhanced, notably even in apparently normal vessels, in the heart of LOXtg/apoEKO, indicating LOX-1 activates endothelial cells under the condition of hyperlipidemia. The increased infiltration of macrophages in LOXtg/apoEKO further supports the effect of LOX-1 on the vascular adhesiveness of leukocytes.

Atheroma formation can be understood as a downstream event of endothelial dysfunction. The formation of atheroma-

like lesions was dramatically accelerated in LOXtg/apoEKO compared with apoEKO. This suggests the overall impact of the LOX-1 functions described above on oxidative stress, induction of adhesion molecules, and the binding of OxLDL and leukocytes is to promote atherosclerosis-like vasculopathic changes. In the analyses of the aortic atherosclerotic lesion, where the expression of the transgene was very limited both before and after high-fat diet, in the mouse lines used the size of the lesion did not reach a statistically significant difference between the apoEKO and LOXtg/apoEKO strains. This is in good agreement with the low expression level of the LOX-1 transgene and further supports a role for LOX-1 in the current model of atherogenesis. Interestingly, most atheromatous lesions were found in intramyocardial vessels, although the epicardial coronary arteries are the most common region of atherogenesis in humans. This might be related to the expression profile of the LOX-1 transgene, or the higher oxidative stress in the region. It is reported that the cholate-containing diets are associated with toxicity and inflammation. This needs to be considered in interpreting the data in the present study. Such toxic and proinflammtory effects might cooperatively act, in the present study, with the proinflammatory nature of LOX-1, hence promoting lipoprotein-mediated localized arteritis.

Based on the data from experimental animal models including the present study, it is possible that LOX-1 promotes endothelial dysfunction and atherosclerosis in humans. Recently, other researchers along with our laboratory have reported that LOX-1 gene polymorphisms are associated with both the progression of atherosclerosis and the incidence of ischemic heart diseases.[34–36] Although these studies were not able to specify whether the disease-related polymorphism of the LOX-1 gene specifically enhances the activity of LOX-1, together with the data reported here, there is support for a proatherogenic effect of LOX-1 in humans. LOX-1 appears to enhance vasculopathic and atherogenic changes through its proinflammatory and prooxidative properties.

In summary, it is demonstrated that LOX-1 enhanced both the inflammatory response and lipid deposition in heart vessels when overexpressed in apoEKO mice. LOXtg/apoEKO should prove to be a useful model for further investigation into the initiation and progression of atheromatous changes in the mouse heart, and in time lead to the development of a mouse model of ischemic heart disease.

Acknowledgments

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Adhesion Molecule Expression in Fibroblasts

Alteration in Fibroblast Biology After Transfection With LOX-1 Plasmids

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Abstract—The endothelial lectinlike, oxidatively (ox-) modified LDL receptor LOX-1 is a critical player in the pathogenesis of atherosclerosis and myocardial ischemia. Ox-LDL binding of LOX-1 results in the expression of various adhesion molecules, which attract monocytes to endothelial cells, an initial step in atherogenesis. We wished to examine the role of the ox-LDL/LOX-1 signaling pathway in fibroblasts, which naturally express low levels of LOX-1. Rat cardiac fibroblasts were transfected with either cytomegalovirus (CMV)-LOX-1^{wt} (amino acids [aa] 1 to 273) or CMV-LOX-$1^{1\text{-}261}$ (an ox-LDL–binding negative mutant, aa 1 to 261) plasmid. Western blots showed that LOX-1 protein expression was increased significantly in cells transfected with CMV-LOX-1^{wt} or CMV-LOX-$1^{1\text{-}261}$ plasmid ($P<0.01$ vs control). Fibroblasts transfected with CMV-LOX-1^{wt} showed ox-LDL binding, whereas fibroblasts without transfection and those transfected with CMV-LOX-$1^{1\text{-}261}$ did not bind ox-LDL. Compared with untransfected cells, ox-LDL treatment (50 μg/mL, 24 hours) markedly induced the expression of the leukocyte adhesion molecules intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM)-1 as well as matrix metalloproteinase (MMP)-1 in cells transfected with CMV-LOX-1^{wt} ($P<0.05$) but not in cells transfected with CMV-LOX-$1^{1\text{-}261}$. Concurrently, ox-LDL treatment enhanced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) ($P<0.05$ vs control) in CMV-LOX-1^{wt}–transfected cells. These data suggest that in cardiac fibroblasts, ox-LDL binds to LOX-1 and activates p38 MAPK, followed by the expression of ICAM-1, VCAM-1, and MMP-1. Thus, fibroblasts transform into an endothelial phenotype on transfection with CMV-LOX-1^{wt} and subsequent exposure to ox-LDL. This study provides a useful model system (plasmid-transfected fibroblasts) to study the molecular biology of LOX-1. ***(Hypertension*. 2005;46:622-627.)**

Key Words: lipoproteins ■ fibroblasts ■ collagen ■ genetics

Oxidatively modified low-density lipoprotein (ox-LDL) plays an important role in the pathogenesis of atherosclerosis.[1,2] Ox-LDL influences the properties of various cells mainly by binding to cell surface receptors.[3,4] Several ox-LDL receptors, including scavenger receptor (SR)-A I/II, CD36, SR-BI, FcγRII, lectinlike ox-LDL receptor-1 (LOX-1), and macrosialin, have been identified in macrophages, smooth muscle cells, endothelial cells, and platelets.[3,5–8] Among them, LOX-1 has received extensive attention because its expression is increased in several pathologic conditions, such as hypertension, diabetes, and atherosclerosis.[9–13]

LOX-1 is mainly expressed in vascular endothelial cells and acts as a cell surface receptor for the binding, internalization, and degradation of ox-LDL.[13] It is also an important target of therapeutic interventions because proatherogenic conditions such as diabetes and hypertension induce its expression.[12,14] In addition, the expression of monocyte adhesion molecules, which are closely associated with atherogenesis, is upregulated by ox-LDL/LOX-1 activation.[15,16]

Fibroblasts produce collagens, which are involved in thickening of the intima of atherosclerotic lesions.[17,18] Fibroblasts also release matrix metalloproteinases (MMPs),[19] which are relevant to plaque disruption and acute ischemic syndromes.[20] As such, fibroblasts are involved in cardiac remodeling by producing collagens as well MMPs.[21] A balance between collagen synthesis and degradation determines plaque stability and vascular and myocardial stiffness. Our previous studies showed extensive expression of LOX-1 in atherosclerotic lesions[22] as well as in the ischemic myocardium.[23] We have also shown that ox-LDL/LOX-1 activation stimulates MMP-1 and MMP-3 expression in endothelial cells.[24] However, the role of LOX-1 in fibroblasts in response to ox-LDL has not yet been studied.

In this study, we cloned the plasmids expressing wild-type LOX-1 (cytomegalovirus [CMV]-LOX-1^{wt}) or the ox-LDL–binding negative-mutant LOX-1, transfected them into rat cardiac fibroblasts, and examined the biologic response of these cells to ox-LDL treatment.

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Methods

Construction of LOX-1 Expression Plasmids

Human LOX-1 cDNA was amplified by polymerase chain reaction (PCR). For wt LOX-1 (273 amino acid residues), the primers were 5′-CGT GAC TGC TTC ACT CTC TCA-3′ (forward) and 5′-TTG GCA CCC AAG TGA CAA A-3′ (reverse); for the ox-LDL–binding negative-mutant LOX-1 (261 amino acid residues, *C*-terminal deletion), the primers were 5′-CGT GAC TGC TTC ACT CTC TCA-3′ (forward) and 5′-CTA GAA GGC AGC TAA AAT GCA G-3′ (reverse). The cDNA was cloned into the pCMVTnTTM plasmid (Promega) with the use of *Eco*RI and *Xba*I restriction enzymes, and the DNA sequence was analyzed by the DNA Sequencing Laboratory at the University of Arkansas for Medical Sciences. No mutation was found. Hereafter, the plasmids expressing wt and mutant LOX-1 are referred to as CMV-LOX-1^{wt} and CMV-LOX-$1^{1\text{-}261}$, respectively.

Cell Culture and Transient Transfection

Rat cardiac fibroblasts were cultured as described earlier.[21] The cells (fifth passage) were plated in 58-mm dishes (Nunchrand) with 5 mL of culture medium. On reaching ≈70% confluence, cells were transfected with CMV-LOX-1^{wt} or CMV-LOX-$1^{1\text{-}261}$ with FuGENE 6 transfection reagent (Roche), according to the manufacturer's suggestions. Five hours after transfection, the medium was replaced with fresh, serum-free medium, and the cells were starved for 48 hours before they were cultured in the complete culture medium with or without 50 μg/mL ox-LDL for 24 hours. Cells then were harvested and subjected to the studies described next.

Binding of DiI–Ox-LDL to Cardiac Fibroblasts

Forty-eight hours after transfection, the cells were incubated with 5 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled ox-LDL (Intracel Resources) in serum-free Dulbecco's modified Eagle's medium on ice for 1 hour and washed 5 times with phosphate-buffered saline. The fluorescence of DiI was observed with a fluorescence microscope.

RNA Extraction and Semiquantitative RT-PCR Analysis

After treatment, total cellular RNA was isolated from fibroblasts with the use of an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Equal amount of total RNA (3 μg) was reverse-transcribed (RT) with oligo(dT) and M-MLV reverse transcriptase (Promega) at 37°C for 1 hour. PCR amplification of rat procollagen type I, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and β-actin was achieved with the use of specific primers. 5′-ACA GCA CGC TTG TGG AT-3′ (forward) and 5′-GTC TTC AAG CAA GAG GAC CA-3′ (reverse) were for procollagen type I; 5′-CTG TCG GTG CTC AGG TAT CC-3′ (forward) and 5′-CCA ACT TCT CAG TCA CCT CC-3′ (reverse) were for ICAM-1; 5′-CGC TCG CTC AGA TTG GAG AC-3′ (forward) and 5′-CTC GCT GGC ACA TGT CAT CG-3′ (reverse) were for VCAM-1; and 5′-GAG CTA TGA GCT GCC TGA CG-3′ (forward) and 5′-AGC ACT TGC GGT CCA CGA TG-3′ (reverse) were for β-actin. PCR was performed in a volume of 50 μL containing 2 μL cDNA mixture, 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 200 mmol/L dNTP, 1.25 U *Taq* polymerase, and 20 pmol/L primers. PCR was carried out with an initial 5-minute denaturation at 94°C, followed by 32 cycles of amplification (60 seconds at 94°C, 50 seconds at 60°C, 2 minutes at 72°C) and a final incubation of 10 minutes at 72°C. The PCR-amplified products were visualized in 1.5% agarose gels containing ethidium bromide. The relative intensity of all bands was normalized against the β-actin bands, and the results were analyzed with UN-SCAN-IT gel software (Silk Scientific).

Western Blot Analysis

After treatment, fibroblasts were harvested and lysed in cell lysis buffer (Cell Signaling Technology). Lysates were centrifuged at 12 000 rpm for 20 minutes at 4°C, and the supernatants were collected. After protein concentration was measured with the Bio-Rad protein assay system, 50 μg protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies directed against procollagen type I (Santa Cruz), MMP-1 (Oncogene), ICAM-1 (Santa Cruz), VCAM-1 (Santa Cruz), LOX-1, and β-actin (Sigma). Membranes were then washed and incubated with horseradish peroxidase–conjugated secondary antibody (Calbiochem), and the protein bands were detected with enhanced chemiluminescence reagents (Amersham).

Data Analysis

All experiments were repeated at least 3 times on different occasions. The data were expressed as mean±SD. Statistical significance was determined in multiple comparisons among groups of data in which ANOVA and Student's *t* test indicated the presence of a significant difference. A value of $P<0.05$ was considered significant.

Results

Binding of DiI–ox-LDL and Expression of LOX-1 in Cardiac Fibroblasts

The expression of LOX-1 in CMV-LOX-1^{wt}– or CMV-LOX-$1^{1\text{-}261}$–transfected cardiac fibroblasts was determined by ox-LDL binding and Western blot analysis. We found that in the basal state, fibroblasts (control) showed negligible ox-LDL binding, which was increased (to ≈50% of total cells) in CMV-LOX-1^{wt}–transfected cells, whereas there was no ox-LDL binding in CMV-LOX-$1^{1\text{-}261}$–transfected cells (Figure 1, top). The transfection efficiency (≈50%) was suggested by transfection of fibroblasts with green fluorescent protein (GFP) expression plasmids and observation by fluorescence microscopy (Figure 1, top).

LOX-1 protein expression under basal conditions or after ox-LDL treatment was relatively modest but increased significantly in CMV-LOX-1^{wt}– as well as in CMV-LOX-$1^{1\text{-}261}$–transfected fibroblasts (both $P<0.01$ vs control). The expression of LOX-1 in fibroblasts under basal conditions and after ox-LDL treatment was similar (Figure 1, bottom) and was much higher after CMV-LOX-1^{wt} or CMV-LOX-$1^{1\text{-}261}$ transfection. We believe that we successfully constructed the LOX-1 expression plasmids and transfected fibroblasts with these plasmids, and this process resulted in expression of LOX-1 in fibroblasts.

Expression of Procollagen Type I and MMP-1 in Cardiac Fibroblasts

To examine the effects of LOX-1 transfection on cardiac fibroblasts, we examined the expression of procollagen type I and MMP-1. The expression of procollagen type 1 (mRNA and protein) was similar in untransfected and both CMV-LOX-1^{wt}– and CMV-LOX-$1^{1\text{-}261}$–transfected cells and did not change after ox-LDL treatment (Figure 2, left). MMP-1 protein expression was also similar in the untransfected and CMV-LOX-1^{wt}– or CMV-LOX-$1^{1\text{-}261}$–transfected cells. However, after ox-LDL treatment, MMP-1 expression increased dramatically in CMV-LOX-1^{wt}–transfected cells ($P<0.01$ compared with untransfected cells), whereas it remained unchanged in CMV-LOX-$1^{1\text{-}261}$–transfected cells (Figure 2, right).

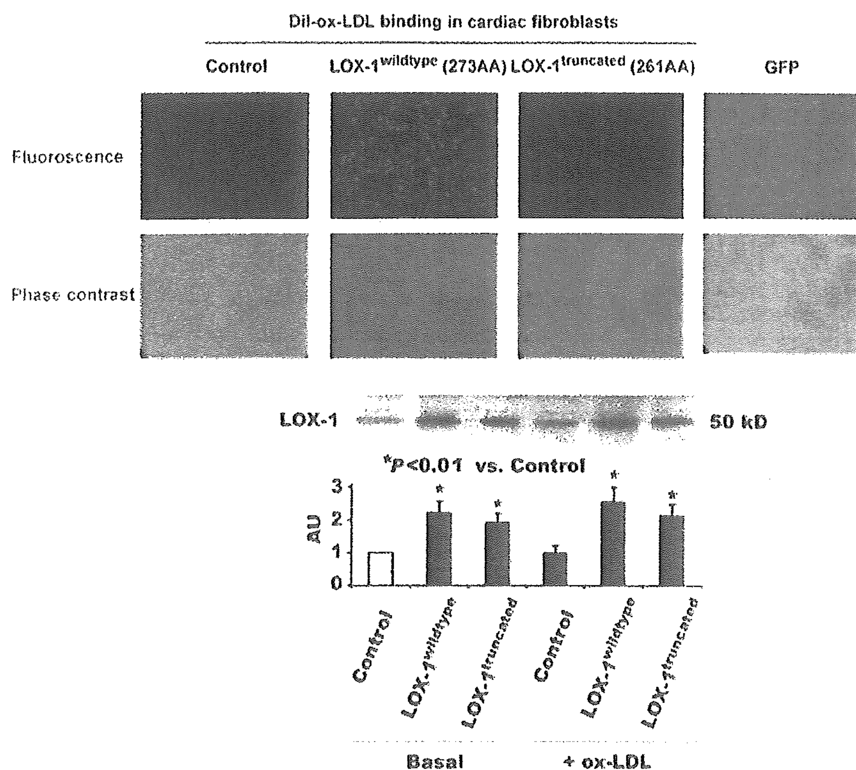

Figure 1. The binding activity of DiI–ox-LDL and the expression of LOX-1. Top, The binding of DiI–ox-LDL was undetected in untransfected control cells but was increased to ≈50% of total cells in CMV-LOX-1^{wt}–transfected cells. It was consistent with the percentage of cells that express GFP after they were transfected with CMV-GFP plasmid. In contrast, binding activity was negligible in CMV-LOX-1$^{1-261}$–transfected cells. The upper panels are representative images captured during fluorescence microscopy, and the bottom panels are representative images from phase-contrast microscopy. Bottom, Western blotting showed that LOX-1 protein expression was low in control cells but was increased markedly in CMV-LOX-1^{wt}– or CMV-LOX-1$^{1-261}$–transfected cells. Abbreviations are as defined in text.

Expression of ICAM-1 and VCAM-1 in Cardiac Fibroblasts

Under basal conditions, expression (mRNA and protein) of ICAM-1 and VCAM-1 was low and remained low after transfection of fibroblasts with CMV-LOX-1^{wt} or CMV-LOX-1$^{1-261}$ (Figure 3). This observation is consistent with the studies by Kacimi et al.[25] who showed minimal expression of ICAM-1 and VCAM-1 in cardiac fibroblasts. We wondered whether LOX-1 overexpression would alter the biology of these cells. We found that transfection with CMV-LOX-1^{wt} or CMV-LOX-1$^{1-261}$ had no effect on the expression of ICAM-1 or VCAM-1 in the basal state. However, after ox-LDL treatment, the expression of both ICAM-1 and VCAM-1 was significantly upregulated in the CMV-LOX-1^{wt}–transfected fibroblasts ($P<0.05$). The expression of both mRNA and protein was increased in CMV-LOX-1^{wt}–transfected fibroblasts. Note that there was no increase in ICAM-1 or VCAM-1 expression in fibroblasts transfected with CMV-LOX-1$^{1-261}$, even after the cells were treated with ox-LDL (Figure 3).

p38 MAPK and p44/42 MAPK Activation in Cardiac Fibroblasts

Mitogen-activated protein kinase (MAPK) is an important intracellular signaling molecule and is activated during ox-LDL/LOX-1 activation in endothelial cells.[15] We examined whether LOX-1 overexpression would affect MAPK activation in fibroblasts. We found that there was no difference in p38 MAPK expression and phosphorylation between transfected and untransfected cells. After ox-LDL treatment, p38 MAPK expression was also similar between transfected and untransfected cells. However, compared with that in untransfected cells, the phosphorylation of p38 MAPK was greatly stimulated in CMV-LOX-1^{wt}–transfected cells ($P<0.05$) but not in CMV-LOX-1$^{1-261}$–transfected cells (Figure 4). Note that the expression and phosphorylation of p44/42 MAPK re-

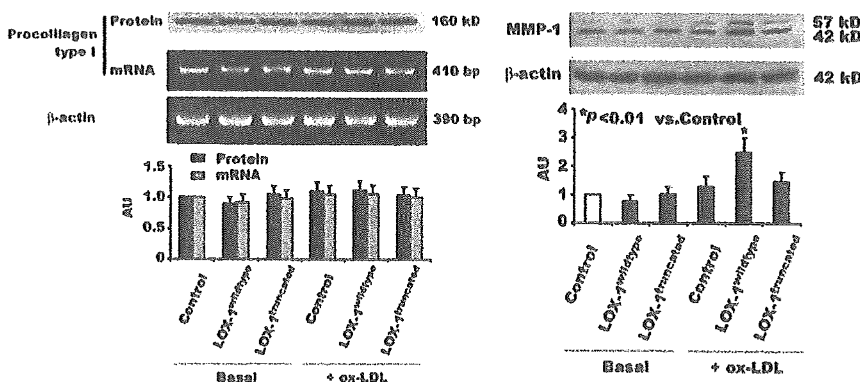

Figure 2. The expression of procollagen type I and MMP-1. Left, Western blot and RT-PCR showed that the expression of procollagen type I was similar in untransfected, CMV-LOX-1^{wt}–transfected, or CMV-LOX-1$^{1-261}$–transfected cells and was not changed after ox-LDL treatment. Right, Western blotting showed that the expression of MMP-1 was similar in untransfected, CMV-LOX-1^{wt}–transfected, or CMV-LOX-1$^{1-261}$–transfected cells under basal conditions. However, after ox-LDL treatment, MMP-1 expression was increased markedly in CMV-LOX-1^{wt}–transfected cells, but it remained unchanged in CMV-LOX-1$^{1-261}$–transfected cells. Abbreviations are as defined in text.

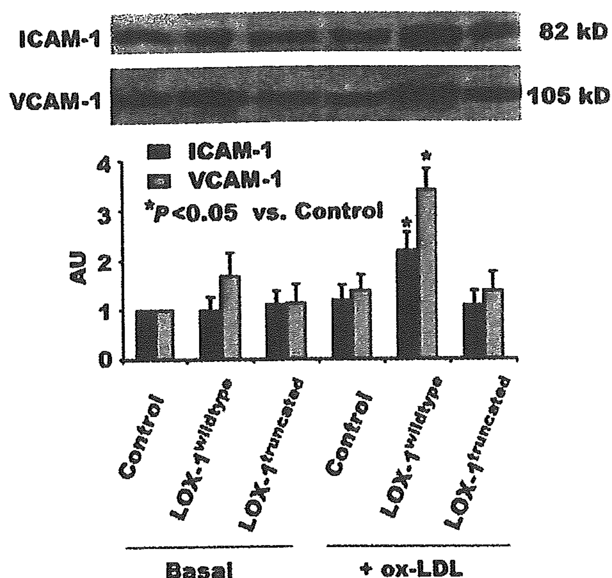

Figure 3. The expression of ICAM-1 and VCAM-1. Expression (mRNA and protein) of ICAM-1 and VCAM-1 was similar in untransfected (control), CMV-LOX-1^{wt}–transfected, or CMV-LOX-$1^{1\text{-}261}$–transfected cells under basal conditions. However, after ox-LDL treatment, expression of ICAM-1 and VCAM-1 (both protein and mRNA) increased markedly in CMV-LOX-1^{wt}–transfected cells, but it remained unaffected in CMV-LOX-$1^{1\text{-}261}$–transfected cells. Abbreviations are as defined in text.

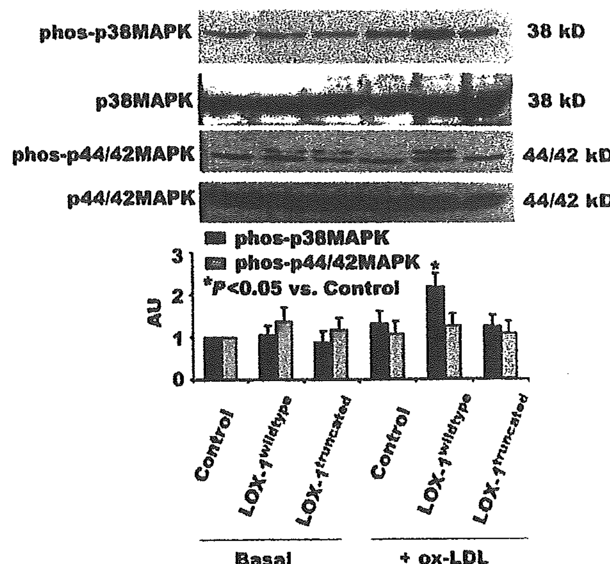

Figure 4. p38 and p44/42 MAPK activation. Western blotting showed that there was no difference in p38 and p44/42 MAPK expression among different groups of cells. However, after ox-LDL treatment, compared with that in untransfected cells, phosphorylation of p38 MAPK was greatly stimulated in CMV-LOX-1^{wt}–transfected cells but not in CMV-LOX-$1^{1\text{-}261}$–transfected cells. In contrast, the phosphorylation of p44/42 MAPK remained unaffected by ox-LDL treatment in these cells.

mained unaffected by CMV-LOX-1^{wt} or CMV-LOX-$1^{1\text{-}261}$ transfection and were unaffected by ox-LDL treatment (Figure 4).

Discussion

This study demonstrates a novel yet undefined alteration in the biology of cardiac fibroblasts, in that LOX-1^{wt}–transfected cells can express high levels of leukocyte adhesion molecules in response to ox-LDL. Furthermore, we have shown that fibroblasts can be used successfully to study LOX-1 mutants and their altered molecular biology. The key to this system is that fibroblasts express only a small amount of LOX-1 under basal conditions, and they can be adequately transfected with wt and mutant LOX-1 expression plasmids.

Transfection of Cardiac Fibroblasts With CMV-LOX-1^{wt} and CMV-LOX-$1^{1\text{-}261}$

Dr Sawamura's group first cloned human LOX-1 cDNA and described that LOX-1 binds, internalizes, and degrades ox-LDL in COS-7 cells.[13] We constructed a wt LOX-1 (aa 1 to 273) expression plasmid (CMV-LOX-1^{wt}) and an ox-LDL–binding negative-mutant LOX-1 (aa 1 to 261) expression plasmid (CMV-LOX-$1^{1\text{-}261}$). In this study, the wt and mutant LOX-1 expression plasmids were successfully transfected into rat cardiac fibroblasts. It was confirmed by the finding that both CMV-LOX-1^{wt}– and CMV-LOX-$1^{1\text{-}261}$–transfected fibroblasts expressed a large amount of LOX-1, whereas untransfected fibroblasts expressed only low levels of LOX-1 (Figure 1, bottom). Furthermore, after transfection, the percentage of cells (CMV-LOX-1^{wt} transfected) that bound ox-LDL was similar to that expressing GFP (CMV-GFP transfected), which served as a transfection efficiency marker (Figure 1, top).

This study has also demonstrated the requirement of the last 12 aa residues at the *C*-terminus of LOX-1 for ox-LDL recognition and binding, in keeping with the results of an earlier study.[26] We confirmed this phenomenon by showing that only cells overexpressing wt LOX-1 (aa 1 to 273), but not the mutant LOX-1 (aa 1 to 261), could bind DiI–ox-LDL. After ox-LDL treatment, expression of MMP-1, ICAM-1, and VCAM-1 was significantly increased in fibroblasts transfected with CMV-LOX-1^{wt} but not in those transfected with CMV-LOX-$1^{1\text{-}261}$. In addition, we found that ox-LDL treatment increased the phosphorylation of p38 MAPK, but not of p44/42 MAPK, in fibroblasts transfected with CMV-LOX-1^{wt} but not with CMV-LOX-$1^{1\text{-}261}$. Taken together, these data suggest that the binding of ox-LDL to LOX-1 is required for its effects to manifest in fibroblasts.

Biologic Function of LOX-1 in Cardiac Fibroblasts

Cardiac fibroblasts are the main source of collagen type I, which accumulates in the heart and leads to fibrosis and heart failure.[21,27,28] Cardiac fibroblasts also release MMP-1 that degrades collagen type I. Under physiologic conditions, there is a balance between collagen type I production and degradation. However, this balance could be disrupted by several pathologic conditions. For example, anoxia/reoxygenation stimulates the expression of both collagen type I and MMP-1,[29] whereas angiotensin II enhances the expression of collagen type I but inhibits the expression of MMP-1.[21] In the present study, we examined whether ox-LDL/LOX-1 activation would influence the expression of collagen type I and MMP-1 in cardiac fibroblasts and found that ox-LDL had no

effect on procollagen type I expression in untransfected, CMV-LOX-1^{wt}–transfected, or CMV-LOX-1$^{1-261}$–transfected fibroblasts. However, LOX-1 upregulation (via plasmid transfection), together with ox-LDL treatment, increased MMP-1 expression markedly. It has been suggested that the release of MMPs is a major factor by which the fibrous cap in atherosclerotic regions softens and finally dissolves, leading to platelet accumulation.[30,31] Ox-LDL accumulation and LOX-1 upregulation have been found in rupture-prone atherosclerotic plaques.[22,32] We showed earlier that ox-LDL–treated endothelial cells release large amounts of MMP-1 and MMP-3 and that this effect of ox-LDL can be blocked by pretreatment of the cells with LOX-1 antibody, suggesting the role of LOX-1.[24] In this article, we have shown that ox-LDL can also increase MMP-1 expression in fibroblasts via LOX-1.

Normally, fibroblasts express only low levels of the leukocyte adhesion molecules ICAM-1 and VCAM-1, even after ox-LDL treatment. In this study, we showed that fibroblasts transfected with CMV-LOX-1^{wt}, but not CMV-LOX-1$^{1-261}$, expressed high levels of ICAM-1 and VCAM-1, both mRNA and protein, in response to ox-LDL. This alteration in the biology of fibroblasts gives them the functional characteristics of endothelial cells.

Our observations gain support from a study by Yoshikawa et al,[33] who showed that tumor necrosis factor-α– and interleukin-4–stimulated fibroblasts facilitate the accumulation of monocytes at inflammation sites. In our preliminary studies in high-cholesterol-diet–fed, LDL-receptor–knockout mice, we found well-developed atherosclerosis and intense expression of CD68 throughout the thickness of the aorta, implying that monocyte/macrophage adhesion is present not just in the subintima but also in the adventitial region, where fibroblasts are present. All of these regions also express high levels of LOX-1 (authors' unpublished data).

MAPK is considered a common intracellular signaling molecule that responds to extracellular stimuli.[34,35] We examined the protein expression and phosphorylation of both p38 and p44/42 isoforms and found that p38 MAPK is involved in the ox-LDL–induced upregulation of MMP-1, ICAM-1, and VCAM-1 in CMV-LOX-1^{wt}–transfected fibroblasts. In previous studies,[21,36,37] we showed that ox-LDL increases the expression of adhesion molecules in endothelial cells via p38 MAPK and nuclear factor-κB activation. Furthermore, inhibition of p38 MAPK abolished these ox-LDL–induced effects. Here we have demonstrated a potentially important role for p38 MAPK activation in a series of reactions as a result of ox-LDL binding to LOX-1 in fibroblasts.

In summary, we have shown in the present study that both CMV-LOX-1^{wt} and CMV- LOX-1$^{1-261}$ plasmids can be successfully transfected into cardiac fibroblasts. The transfection of CMV-LOX-1^{wt} results in a functional alteration and transduction pathway activation in cardiac fibroblasts similar to those in endothelial cells in response to ox-LDL. Although LOX-1 expression increased in cells transfected with either CMV-LOX-1^{wt} or CMV-LOX-1$^{1-261}$, only CMV-LOX-1^{wt} transfection resulted in the binding of DiI–ox-LDL. Furthermore, ox-LDL treatment only increased the expression of MMP-1, ICAM-1, and VCAM-1 and the activation of p38 MAPK in CMV-LOX-1^{wt}–transfected cells.

Perspective

Understanding fibroblast function in vascular biology is gaining renewed attention. These cells play a potent role in atherosclerosis, in both plaque rupture and vascular remodeling, including the formation of aneurysms. These cells also have an important role in cardiac remodeling as a result of ischemic and nonischemic cardiomyopathy. All of these conditions are associated with a marked increase in oxidative stress. The alterations in fibroblast function described herein provide a novel link between oxidative stress, ox-LDL, and LOX-1 in the biology of fibroblasts. Furthermore, these studies show that fibroblasts are a valuable system for studying the molecular biology and function LOX-1.

Acknowledgments

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Acute Coronary Syndrome

Enhanced Plasma Levels of Oxidized Low-Density Lipoprotein Increase Circulating Nuclear Factor-Kappa B Activation in Patients With Unstable Angina

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OBJECTIVES The purpose of this study was to investigate the effect of circulating levels of oxidized low-density lipoprotein (ox-LDL) on nuclear factor-kappa B (NF-kB) activation in peripheral blood mononuclear cells (PBMC) of patients with unstable angina (UA) or stable angina (SA) and control subjects.

BACKGROUND Nuclear factor-kB might be involved in atherosclerosis, as is suggested by the presence of activated NF-kB in human atherosclerotic lesions.

METHODS Levels of plasma ox-LDL and circulating NF-kB in PBMC (and in separated lymphocytes and monocytes) were measured in 27 control subjects and 29 SA and 27 UA patients. In in vitro studies, the effect of ox-LDL and of the sera derived from a subgroup of UA patients and control subjects on monocytic NF-kB activation was also evaluated.

RESULTS The UA and SA patients had higher levels of circulating ox-LDL and NF-kB in PBMC than control subjects ($p < 0.001$). The increase in circulating NF-kB was mainly due to the activation of monocytes. In the in vitro studies, ox-LDL dose-dependently increased the activation of NF-kB in monocytes, but not in lymphocytes derived from healthy volunteers. This increase was related to the expression of lectin-like ox-LDL receptor-1 on monocytes. The incubation of monocytes with the sera derived from the UA patients induced a significant increase in NF-kB activation compared with the sera derived from the control subjects.

CONCLUSIONS The data suggest that the activation of NF-kB in monocytes of UA patients is, at least in part, induced by circulating molecules such as ox-LDL, which has been found to be particularly elevated in UA patients. (J Am Coll Cardiol 2005;46:799–806) © 2005 by the American College of Cardiology Foundation

There is increasing evidence that inflammation plays an important role in atherogenesis and might determine plaque vulnerability (1). Many of the genes involved in the acute inflammatory response that are pivotal in the atherogenic process are activated by the nuclear factor-kappa B (NF-kB) (2). Nuclear factor-kB resides inactive and bound to the inhibitory protein-kappa B (I-kB) in the cytoplasm of many cell types, including T-lymphocytes, monocytes, macrophages, endothelial cells, and smooth muscle cells (3,4). Numerous stimulants—including cytokines and oxidants (5) such as oxidized low-density lipoprotein (ox-LDL) (6)—alter I-kB, causing nuclear translocation of NF-kB.

Nuclear factor-kB was demonstrated to be present in human coronary plaque and enhanced in patients with unstable angina (UA) (7,8). Recent work has shown an association between increasing circulating level of NF-kB in peripheral blood mononuclear cells (PBMC) and UA (8). So far, it is unclear whether NF-kB activation in the circulation of patients with UA represents cause or effect, with peripheral activation of the transcription factor occurring in response to a stimulus in the coronary circulation or the coronary event itself occurring as a reaction to circulating inflammation.

Recent data indicate that circulating levels of ox-LDL are high in acute coronary syndromes (ACS) and, in particular, in UA (9). The mechanisms leading to this increase are unclear. However, it is known that plaque instability correlates with the location of macrophages, T cells, and mast cells within the plaque (1). Moreover, macrophage-rich plaques have been recently shown to contain higher concentration of ox-LDL than macrophage-poor plaques (10,11) and to be associated with elevated levels of ox-LDL in plasma (10). Taken together, this evidence suggests that plasma and plaque levels of ox-LDL may be correlated with the vulnerability to rupture of atherosclerotic lesions.

There are many scavenger receptors that can bind ox-LDL (12). Currently, however, the majority of these recep-

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Abbreviations and Acronyms

ACS	= acute coronary syndrome
CAD	= coronary artery disease
HDL	= high-density lipoprotein
IgG	= immunoglobulin G
LOX-1	= lectin-like ox-LDL receptor-1
LPDS	= lipoprotein-depleted serum
mAb	= monoclonal antibody
mRNA	= messenger ribonucleic acid
NF-kB	= nuclear factor-kappa B
ox-LDL	= oxidized low-density lipoprotein
PBMC	= peripheral blood mononuclear cells
PCI	= percutaneous coronary intervention
RT-PCR	= reverse transcriptase-polymerase chain reaction
SA	= stable angina
UA	= unstable angina

tors have been shown to be mostly non-signaling molecules and to play a key role in foam cell formation (13). In contrast, ox-LDL/lectin-like ox-LDL receptor-1 (LOX-1) interaction has already been shown to modulate cellular function (14) and to induce NF-kB activation in endothelial cells (15).

Therefore, the aim of this study was to assess the role of plasma ox-LDL and LOX-1 on circulating NF-kB activation in patients with UA.

METHODS

Study population. This study was approved by our hospital ethical committees, and informed consent was obtained from all patients before their enrolment.

Major requirements for enrollment in all the groups were: absence of infectious or acute/chronic inflammatory diseases, known malignancy, absence of acute/chronic renal failure, or hepatic failure. Three groups of patients were studied.

STABLE ANGINA (SA) GROUP. This group comprised patients with typical effort anginal pain associated with documentation of inducible ischemia during exercise stress test, defined as ST-segment depression >1 mm on the electrocardiogram during bicycle ergometry or ipo/akinesia of one or more normocontractile segments of the cardiac wall during stress-echo; no significant worsening of the symptoms in the previous two months; and no anginal episodes in the week before enrollment.

UA GROUP. This group comprised patients with at least two episodes of rest anginal pain or one episode lasting more than 20 min in the previous 48 h (class IIIB unstable angina, according to Braunwald's classification) (16), preferably, but not necessarily, associated with electrocardiographic modifications (T-wave inversion, ST-segment depression, transient ST-segment elevation) and a normal value of I-troponin on admission and during the first 24 h.

For both SA and UA patients, exclusion criteria were: previous coronary artery bypass grafting, recent (<6 months) myocardial infarction, recent (<6 months) percutaneous coronary intervention (PCI), congestive heart failure, and coronary tree free of significant coronary artery disease (CAD) (defined as at least one stenosis with minimal luminal diameter >70% of the arterial lumen by visual estimate) detected at coronary angiography.

CONTROL GROUP. This group comprised patients affected by valvular or congenital heart disease, but without a clinical history of CAD, who underwent coronary angiography for a pre-surgical evaluation. The only exclusion criterion was detection of significant CAD during coronary angiography.

The following data were obtained from all patients: age, gender, presence of CAD risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, family history of CAD), use of medications, previous myocardial infarction, and previous revascularization (PCI or coronary artery bypass grafting).

Blood samples. Venous blood samples were obtained from SA and control patients the morning after the day of admission. In UA patients, samples were drawn within 24 h from the index event (the last episode of anginal pain or admission if angina occurred previously). Blood was collected from each patient and drawn into pyrogen-free blood collection tubes without additives. Multiple aliquots of serum were placed into sterile 5-ml screw-capped polypropylene vials and stored at -80°C in plastic bags. Samples were kept frozen for no longer than 30 days, with an average of 21 days. For thawing, serum tubes were placed overnight in a refrigerator at 2°C to 8°C. The presence of flocculent material was removed by aseptic filtration through a sterile 0.45-μm filter. The samples were frozen and thawed only once.

Plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglycerides, glucose, white blood cell count with differential count, and high-sensitivity C-reactive protein were measured with standard techniques used in the Central Laboratory of Verona University Hospital. Levels of plasma ox-LDL and circulating NF-kB in PBMC and in separated lymphocytes and monocytes and of LOX-1 messenger ribonucleic acid (mRNA) and protein expression were measured by investigators blinded to the clinical and angiographic characteristics of the patients.

PBMC and monocyte isolation. Peripheral blood mononuclear cells were separated, as previously described (17), in all subjects enrolled in the study. Isolation of monocytes and lymphocytes was performed in 10 subjects of each group. Monocytes and lymphocytes were isolated from PBMC by negative or positive selection with a cocktail of hapten-conjugated antibodies and magnetic microbeads coupled to an anti-hapten monoclonal antibody (No touch monocyte isolation kit; Miltenyi Biotec, Auburn, California) and depletion on a column in a magnetic field (VarioMACS, Miltenyi Biotec). Monocyte and lymphocyte purity was >97%, as assessed by flow cytometry (data not shown).

Endotoxin contamination of cell cultures, involving the use of ox-LDL and lipoprotein-depleted serum (LPDS), was routinely excluded with the chromogenic limulus amebocyte lysate assay (Sigma, St. Louis, Missouri). Furthermore, all cell cultures were set up in the presence of 10 μg/ml polymixin B to neutralize any potential lipopolysaccharide contamination.

NF-kB measurement. Nuclear factor-kB activation was measured by two methods: 1) by a sensitive multi-well colorimetric assay for active NF-kB (TRANS-AM; Active Motif, Rixensart, Belgium), as previously described (18); and 2) by flow cytometry, to differentiate NF-kB activation in lymphocytes and monocytes. As a reference, recombinant p65 (Active Motif) was used.

Flow cytometric analysis was performed according to a previously published procedure (19). Whole blood cells were labeled with phycoerythrin-conjugated anti-CD14 monoclonal antibodies and peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies. The cells were then labeled with mouse anti–NF-kB (nuclear-localized signal) antibodies (IgG3; Boehringer Mannheim, Mannheim, Germany), recognizing an epitope overlapping the nuclear location signal of NF-k-p65, and therefore, the activated form of NF-kB. The cells were then labeled with fluorescein isothiocyanate-conjugated rat anti-mouse IgG3 monoclonal antibodies (Pharmingen, San Diego, California). Immunofluorescence staining was analyzed with a FACScan flow cytometer, equipped with CellQuest software (Becton-Dickinson Biosciences, San Jose, California).

Oxidized LDL assay. The ox-LDL were measured with the enzyme-linked immunosorbent assay Mercodia Oxidized LDL ELISA kit, in which the wells of the microtiter plates are coated with the capture monoclonal antibody (mAb)-4E6 (9) (Mercodia AB, Uppsala, Sweden), following the method described by Holvoet et al. (20). As a standard solution, Cu^{2+}-modified LDL, ranging from 50 to 500 ng/ml, was used.

LDL isolation and oxidation. Whole blood from healthy volunteers, containing ethylenediamine tetraacetic acid (EDTA) (1 mg/ml), was processed for LDL separation as previously described (21). Cu^{2+}-modified LDL was prepared as previously reported (15).

LOX-1 mRNA and protein expression in separated lymphocytes and monocytes. Separated monocytes and lymphocytes were from pools derived from control subjects and SA and UA patients. Total RNA was extracted from cells by the RNAse mini kit (Qiagen, Venlo, the Netherlands). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with the TwoStep RT-PCR kit (Invitrogen, Carlsbad, California). For each reaction, 1 μg of total RNA served as a template. For amplification, a primer pair specific for human LOX-1 (sense primer, 5′-TTACTCTCCATGGTGGTGCC-3′; antisense primer, and 5′-AGCTTCTTCT DCTTGTTGCC-3′) was used; beta-ACTIN (5′-ATCTGGCACCACACCTTCTAC-3′ and 5′-GAGGCGTACAGGGATAGCAC-3′) was used as an internal standard in the PCR mixture. A 199-bp human LOX-1 complementary DNA (cDNA) fragment and a 182-bp human beta-ACTIN cDNA fragment were enzymatically amplified by 30 and 22 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1.5% Tris-acetate EDTA agarose gel containing ethidium bromide. The intensity of the bands was measured with an image analysis scanning system (Alpha Imager 2000; Packard Instruments, Meriden, Connecticut).

The LOX-1 protein expression was analyzed in monocytes and lymphocytes by flow cytometry, as described previously, using a specific anti-LOX-1 mAb (22).

Oxidized LDL-dependent activation of NF-kB in monocytes. Purified monocytes (3×10^5/ml, 200 μl/well) from healthy donors were cultured in 96-well trays (Costar, Cambridge, Massachusetts) in RPMI 1640 with L-glutamine (GIBCO; Invitrogen) for 20 h at 37°C, with increasing amounts of ox-LDL (from 10- to 40-μg/ml medium as measured by the previously specified enzyme-linked immunosorbent assay).

In some experiments, blocking anti-LOX-1 mAb (20 μg/ml) or control mouse immunoglobulin G (IgG) (50 μg/ml) was also added to cell culture. Nuclear factor-kB was measured in cellular extract of monocytes as previously indicated.

Plasma-dependent activation of NF-kB in monocytes. Purified monocytes (3×10^5/ml, 200 μl/well) from healthy volunteers were cultured in 96-well trays (Costar) in RPMI 1640 with L-glutamine (GIBCO) for 20 h at 37°C with 40% serum from 10 UA patients with the highest ox-LDL levels or control subjects with the lowest ox-LDL values. For processing of serum, 80 μl of serum from each patient or control subject was added to the monocyte culture immediately after thawing, at the start of the culture period.

In some experiments, anti-LOX-1 mAb (20 μg/ml) or control human IgG (50 μg/ml) was also added to the cell culture.

As a further control, monocytes were also incubated with the corresponding LPDS in which all the lipoproteins were taken away by ultracentrifugation at a density >1.21 g/ml, following the previously indicated method (21).

Statistical analysis. Continuous data are expressed as mean ± SD values, if normally distributed. Median (interquartile range) was used for variables not normally distributed. Normal distribution of the data was determined with the Shapiro-Wilk test. Differences between continuous data were analyzed by the two-tailed unpaired Student *t* test. Statistical comparison among three groups was performed by one- or two-way analysis of variance and post-hoc multiple comparison with Student-Newmann-Keuls' test, if a parametric distribution was assessed. If the data were not parametric, analysis of variance on ranks and post-hoc Dunn's tests were used. Relationship between variables was assessed by linear regression. A probability value <0.05 was

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Table 1. Baseline Clinical Characteristics of the Three Groups of Patients

	C (n = 27)	SA (n = 29)	UA (n = 27)	p Value
Age (yrs)	66 ± 11	61 ± 9	61 ± 9	NS
Women	9 (33%)*	3 (10%)	3 (11%)	<0.05
Risk factors				
Family history	0 (37%)	19 (66%)	10 (38%)	NS
Smoke	5 (18%)	8 (28%)	12 (46%)	NS
Hypercholesterolemia	11 (41%)*	23 (79%)	18 (69%)	<0.05
Hypertension	3 (48%)	23 (79%)	16 (61%)	NS
Diabetes	2 (7%)	4 (14%)	4 (15%)	NS
Current therapy				
ACE inhibitors	17 (63%)	17 (59%)	9 (35%)	NS
Statins	3 (11%)	7 (24%)	3 (11%)	NS
Aspirin	8 (30%)	22 (76%)†	13 (50%)	<0.05
Clinical history				
Previous ACS	—	19 (65%)‡	8 (31%)	<0.01
Previous PCI	—	3 (45%)‡	2 (8%)	<0.01

Data are expressed as numbers and percent. *C versus SA and UA; †SA versus C; ‡SA versus UA.
ACE = angiotensin-converting enzyme; ACS = acute coronary syndrome; C = control subjects; PCI = percutaneous coronary intervention; SA = stable angina; UA = unstable angina.

considered statistically significant. All data were analyzed with SPSS 11.01 for Macintosh (SPSS, Chicago, Illinois).

RESULTS

Baseline characteristics of the patients. During a period of 14 months, 102 patients were enrolled in the study; of these patients, only 82 (27 control subjects, 29 with SA, and 27 with UA) fully satisfied the enrollment criteria. Baseline clinical characteristics of the patients are listed in Table 1. In the control group, there were more women and fewer patients with hypercholesterolemia than in the SA and UA groups ($p < 0.05$); in the SA group, there were more patients with a history of ACS or PCI than in the UA group ($p < 0.01$) and more patients taking aspirin than in the control group ($p < 0.05$).

Laboratory data. Data on total, LDL, and HDL cholesterol, triglycerides, glucose plasma levels, white blood cell count, and C-reactive protein are shown in Table 2. There were no significant differences in total, LDL, or HDL cholesterol, triglycerides, or glucose levels; the total white blood cell counts and C-reactive protein levels were higher in UA patients than in SA or control patients ($p < 0.001$).

Circulating ox-LDL and NF-kB. Figures 1A and 1B show the levels of ox-LDL (expressed in μg/ml) and activated NF-kB (expressed in ng/μg cell protein) in the three groups of patients. The ox-LDL level differed significantly among groups. In particular, post-hoc tests revealed a significant difference between UA and SA patients and control subjects and between SA patients and control subjects (p from <0.001 to <0.01). Also, NF-kB in PBMC (Fig. 1B) results were significantly different in the three groups of patients. The UA and SA patients had higher levels of circulating NF-kB than control patients ($p < 0.001$). Furthermore, a statistically significant difference was observed between SA and UA patients ($p < 0.01$).

In the whole population, a statistically significant correlation was detected between ox-LDL in plasma and NF-kB in PBMC ($r = 0.68$, $p < 0.001$) (Fig. 2).

Percentage of CD3+ T cells and CD14+ monocytes exhibiting NF-kB activation. By flow cytometry, there was only a slight increase in the percentage of CD3+ T cells exhibiting NF-kB activation in UA and SA patients and in control subjects, and there were no significant differences among the three groups (Table 3). In contrast, the percentage of CD14+ monocytes exhibiting NF-kB activation was high, and there were significant differences among the three groups (Table 3). In particular, UA and SA patients had a higher percentage of NF-kB activation in CD14+ mono-

Table 2. Laboratory Data of the Three Groups of Patients

	C (n = 27)	SA (n = 29)	UA (n = 26)	p Value
Total cholesterol (mg/dl)	206.1 ± 25.9	206.4 ± 23.4	215.0 ± 27.1	NS
LDL cholesterol (mg/dl)	133.0 ± 26.4	136.5 ± 4.5	139.9 ± 27.4	NS
HDL cholesterol (mg/dl)	49.9 ± 9.42	46.8 ± 10.9	44.4 ± 8.50	NS
Triglycerides (mg/dl)	135.5 ± 69.4	139.9 ± 49.6	155.0 ± 66.2	NS
Glucose (mg/dl)	106.3 ± 25.6	97.3 ± 18.0	100.8 ± 16.2	NS
White blood cells (10^9/l)	6.75 ± 1.57	6.51 ± 1.81	9.07 ± 1.63*	<0.001
CRP (mg/dl)	0.43 (0.08–0.83)	0.51 (0.08–1.21)	0.98 (0.25–2.01)*	<0.001

*UA versus SA and C.
CRP = C-reactive protein; HDL = high-density lipoprotein; LDL = low-density lipoprotein; other abbreviations as in Table 1.

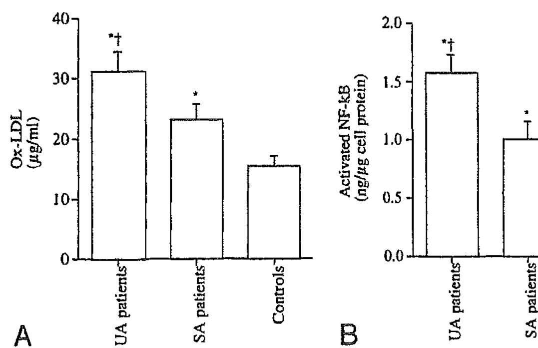

Figure 1. (A) Concentrations of plasma oxidized low-density lipoprotein (Ox-LDL) in unstable angina (UA) and stable angina (SA) patients and in control subjects. *p < 0.001 vs. control subjects; †p < 0.01 vs. SA patients. (B) Concentrations of circulating nuclear factor-kB (NF-kB) in UA and SA patients and in control subjects. The NF-kB was extracted from peripheral blood mononuclear cells derived from UA and SA patients and control subjects. *p < 0.001 vs. control subjects; †p < 0.01 vs. SA patients.

cytes than control patients ($p < 0.001$) (Table 3). A statistically significant difference was also observed between SA and UA patients ($p < 0.01$) (Table 3).

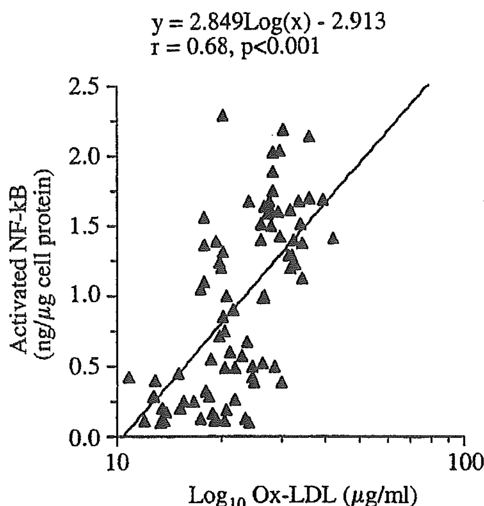

Figure 2. Correlation between the concentrations of plasma oxidized low-density lipoprotein (Ox-LDL) and the circulating levels of nuclear factor-kB (NF-kB) in all subjects considered in the study.

Table 3. Percentage of Cells Exhibiting NF-kB Activation in Patients With Unstable Angina (UA), Stable Angina (SA), and Control Subjects

	CD3+	CD14+
UA patients	4.12 ± 1.83	76.43 ± 8.92*†‡
SA patients	3.41 ± 1.69	44.21 ± 8.21*†
Control subjects	4.10 ± 2.51	7.21 ± 4.13

*p < 0.001 versus CD3+; †p < 0.001 versus control subjects; ‡p < 0.01 versus SA patients.
NF-kB = nuclear factor-kappa B; other abbreviations as in Table 1.

NF-kB activation in separated lymphocytes and monocytes. The results on NF-kB activation in CD14+ monocytes were confirmed by directly measuring NF-kB activation in separated monocytes and lymphocytes derived from a subgroup of UA, SA, and control subjects (Fig. 3). On average, activated NF-kB levels (expressed in ng/μg cell protein) were higher in monocytes than in lymphocytes in UA, SA, and control subjects ($p < 0.001$). There were no differences in NF-kB levels of lymphocytes among the three groups of subjects; conversely, monocytes of UA and SA patients had higher levels of activated NF-kB than control patients ($p < 0.001$). A statistically significant difference was also observed between SA and UA patients ($p < 0.01$).

LOX-1 mRNA and protein expression in monocytes and lymphocytes. To examine whether LOX-1 was expressed in lymphocytes and monocytes, we measured the level of LOX-1 mRNA and protein. Both mRNA and peptide were present in monocytes but not in lymphocytes (Figs. 4A and 4B).

Oxidized LDL-dependent activation of NF-kB in monocytes and lymphocytes. Different amounts of Cu^{2+} ox-LDL (from 10 to 40 μg/ml, as measured in the previously specified assay), incubated overnight with monocytes derived from healthy volunteers, dose-dependently increased NF-kB activation (Fig. 5) ($p < 0.001$). Oxidized LDL had

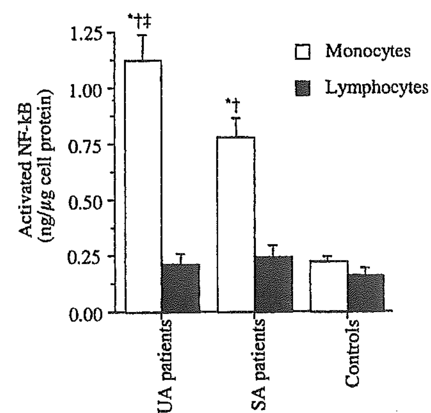

Figure 3. Concentrations of circulating nuclear factor-kB (NF-kB) in separated monocytes and lymphocytes derived from unstable angina (UA) and stable angina (SA) patients and control subjects. The NF-kB was extracted from the separated monocytes and lymphocytes of a subgroup of UA, SA, and control subjects. *p < 0.001 vs. lymphocytes; †p < 0.001 vs. control subjects; ‡p < 0.01 vs. SA patients.

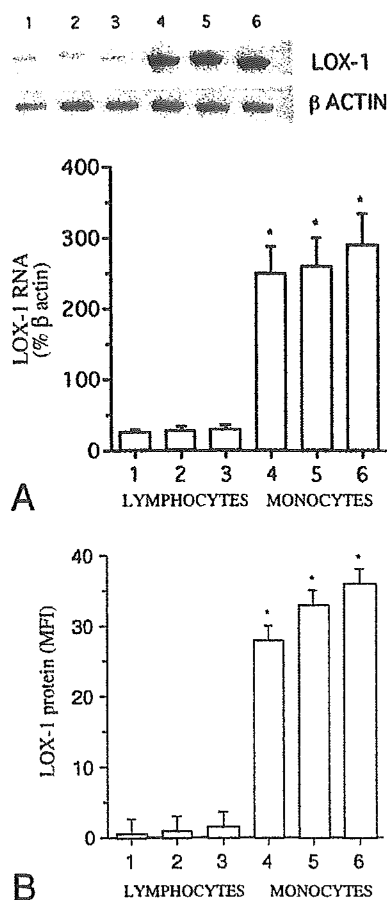

Figure 4. **(A)** Basal lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) messenger ribonucleic acid (mRNA) expression in pools of separated lymphocytes **(1 to 3)** and monocytes **(4 to 6)**, derived from control subjects **(1 and 4)** and stable **(2 and 5)** and unstable **(3 and 6)** angina patients. The LOX-1 mRNA was analyzed by reverse-transcriptase polymerase chain reaction. The LOX-1 mRNA levels were normalized to the levels of beta-actin mRNA. Data illustrated on the bar graph represent the mean ± SD of six different experiments. *$p < 0.001$ vs. lymphocytes **(1 to 3)**. **(B)** The LOX-1 protein expression in pools of separated lymphocytes **(1 to 3)** and monocytes **(4 to 6)** derived from control subjects **(1 and 4)** and stable **(2 and 5)** and unstable **(3 and 6)** angina patients. Lymphocytes and monocytes were separated as described in the Methods section. The LOX-1 protein expression was analyzed by flow cytometry with a specific anti-LOX-1 monoclonal antibody. Results are expressed as mean fluorescence intensity (MFI) and are means ± SD of experiments performed in triplicate on six separate occasions. *$p < 0.001$ vs. lymphocytes **(1 to 3)**.

no effect in lymphocytes (Fig. 5). In some experiments, anti-LOX-1 mAb (20 μg/ml) or control mouse IgG (50 μg/ml) was also added to the cell culture. The presence of the blocking antibody significantly reduced the ox-LDL–dependent activation of NF-kB in monocytes (Fig. 5) ($p < 0.001$).

Plasma-dependent activation of NF-kB in monocytes. Monocytes from healthy volunteers were evaluated for NF-kB activation after they were cultured for 20 h in a medium supplemented with either 40% serum from 10 UA patients with the highest ox-LDL plasma concentrations (range from 41 to 62 μg/ml) or 40% serum from 10 control subjects with the lowest ox-LDL levels (range from 4 to 7 μg/ml). The incubation of monocytes with the sera derived

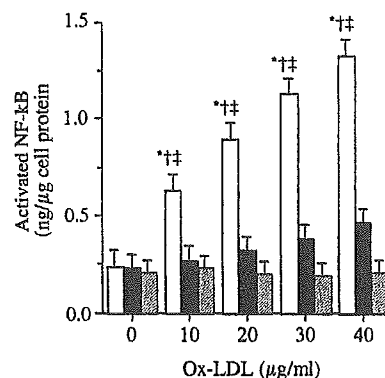

Figure 5. Oxidized low-density lipoprotein (Ox-LDL)-dependent activation of nuclear factor-kB (NF-kB) in monocytes and lymphocytes. The purified monocytes and lymphocytes (3 × 10^5/ml, 200 μl/well) were cultured in 96-well trays for 20 h at 37°C with increasing amounts of Ox-LDL in the presence of control mouse immunoglobulin G (IgG) (50 μg/ml) or anti-LOX-1 monoclonal antibody (mAb) (20 μg/ml). The NF-kB was measured in cellular extract as described in the Methods section. Results are means ± SD of experiments performed in triplicate on six separate occasions. *$p < 0.001$ vs. control value (ox-LDL = 0 mg/ml); †$p < 0.001$ vs. anti-LOX-1 mAb and lymphocytes. **White bars** = Ox-LDL + monocytes + IgG; **black bars** = Ox-LDL + monocytes + anti-LOX-1 mAb; **ruled bars** = Ox-LDL + lymphocytes + IgG.

from the UA patients with the highest values of ox-LDL induced a significant increase in NF-kB activation compared with the sera derived from the control subjects with the lowest values (Fig. 6) ($p < 0.001$).

The NF-kB activation was partially blocked by anti-LOX-1 mAb (Fig. 6) ($p < 0.001$). The incubation of monocytes with the LPDS of UA patients still induced a

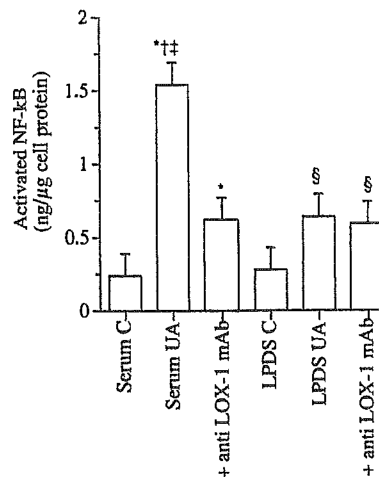

Figure 6. Plasma-dependent activation of nuclear factor-kB (NF-kB) in monocytes. Monocytes from healthy volunteers were evaluated for NF-kB activation after they were cultured for 20 h in medium supplemented with either 40% serum from 10 unstable angina (UA) patients with the highest oxidized low-density lipoprotein (ox-LDL) plasma concentrations (range from 41 to 62 μg/ml) or 40% serum from 10 control subjects with the lowest ox-LDL levels (range from 4 to 7 μg/ml). Monocytes were also incubated with the corresponding lipoprotein-depleted serum (LPDS). In some experiments, anti-lectin–like ox-LDL receptor-1 (LOX-1) monoclonal antibody (mAb) (20μg/ml) or control mouse immunoglobulin G (50 μg/ml) was also added to the cell culture. Results are means ± SD of experiments performed in triplicate on six separate occasions. *$p < 0.001$ vs. serum from control subjects (C); †$p < 0.001$ vs. serum UA + anti-LOX-1 mAb; ‡$p < 0.001$ vs. LPDS UA; §$p < 0.01$ vs. LPDS C.

significant increase of NF-kB compared with the LPDS of the control subjects (Fig. 6). The increase, however, was much lower than that determined by sera in toto ($p < 0.001$) and was not inhibited by anti-LOX-1 mAb (Fig. 6).

DISCUSSION

This study shows that circulating levels of ox-LDL and NF-kB were significantly higher in patients with UA than in SA or control patients. A difference in ox-LDL and activated NF-kB between UA and SA patients was also found.

Several studies have investigated the correlation between clinical manifestations of CAD and circulating levels of ox-LDL. The results of these studies are quite variable, given the heterogeneity of the populations studied and of antibodies used that detect different oxidation-specific epitopes of ox-LDL (9,11,23). In our study, circulating ox-LDL concentrations were measured using the 4E6 antibody that is directed against an epitope generated by the substitution of lysine residues of apoprotein B 100 with aldehydes (9). Holvoet et al. (9) found elevated ox-LDL levels in patients with ACS using the 4E6 antibody, but failed to separate UA and SA patients. These contradictory results might be related to patient selection. In fact, in the study by Holvoet et al. (9), troponin was not measured, and it was not clearly specified whether their UA patients were only in class III or also in classes II or I, according to Braunwald's classification (16).

In this study, we also showed that circulating NF-kB was more elevated in patients with UA than in patients with SA or in control subjects without severe CAD. Our results also show that NF-kB was significantly higher in SA patients than in control subjects. Although for UA patients our results are in agreement with previous findings (8), this is the first demonstration that circulating activated NF-kB is also higher in SA patients than in control subjects, indicating a progressive increase of activated NF-kB from control subjects without severe CAD to UA patients. At variance with our results, Ritchie (8) did not find any difference in circulating NF-kB between SA patients and control subjects. A likely explanation of this discrepancy might be the fact that Ritchie (8) measured NF-kB by electrophoretic mobility shift assay with subsequent densitometric semi-quantitative evaluation of the electrophoretic bands, whereas in this study, circulating NF-kB was evaluated with a sensitive multi-well colorimetric assay that was reported to be at least 10 times more sensitive than electrophoretic mobility shift assay (18).

We then evaluated which cells in PBMC specifically contributed to the increase in circulating NF-kB activation. It was found that the increase in circulating NF-kB activation was mainly due to the activation of monocytes. A number of circulating agents, including cytokines and oxidants (5), or a locally produced substance at the site of plaque rupture might be responsible. In this context, for in vitro experiments, we found that ox-LDL dose-dependently increased the activation of NF-kB in monocytes derived from healthy volunteers. In contrast, ox-LDL had no effect in lymphocytes. These results agree with previous data showing a deregulation of monocytic NF-kB by ox-LDL (6). The increase in monocytic NF-kB activation induced by ox-LDL was related to the expression of LOX-1 on the monocytes, but not on lymphocytes, and was dependent on the binding of ox-LDL to this receptor, because blocking anti-LOX-1 mAb significantly reduced NF-kB activation. The finding that ox-LDL did not activate NF-kB in lymphocytes might, therefore, depend on the fact that, as previously shown (24), LOX-1 mRNA and protein were not expressed in these cells.

Finally, it has to be mentioned that the amounts of ox-LDL that increased monocytic NF-kB activation in these in vitro experiments ranged between 20 and 40 μg/ml medium, as measured with the same enzyme-linked immunosorbent assay used for plasma samples. Thus, they were in the same order of concentration found in the plasma of UA patients.

From these results we then evaluated the effect of sera derived from UA patients and control subjects on NF-kB activation in monocytes derived from healthy volunteers. The results show that the sera of UA patients increased NF-kB activation at a much higher extent than those of control subjects. Because a number of circulating agents could potentially increase monocytic NF-kB activation, the LPDS of UA patients were then used to evaluate the magnitude of lipoprotein effect on NF-kB activation. In these conditions (i.e., in absence of lipoproteins and, therefore, of ox-LDL), the increase in monocytic NF-kB activation was about 70% lower than that obtained with the complete sera. Furthermore, the fact that the contemporary incubation of plasma from UA patients with blocking anti-LOX-1 mAb decreased the NF-kB activation on the same order of magnitude demonstrates that ox-LDL greatly contributes to the activation of NF-kB in monocytes of UA patients.

Taken together, these results show that the activation of NF-kB in monocytes of UA patients might be related to the binding of ox-LDL to LOX-1. These conclusions are in line with the findings that ox-LDL/LOX-1 interaction has already been shown to modulate cellular function (14) and to induce NF-kB activation in endothelial cells (15).

In conclusion, the data of this study suggest that monocytic activation of NF-kB in UA patients is not a primary event, but rather, induced, at least partially, by circulating molecules such as ox-LDL, which have been found to be particularly elevated in UA patients. The activation of NF-kB in these cells might participate in up-regulating the expression of some genes such as interleukin-8, interleukin-1, and tissue factor, proposed to be involved in atherogenesis (5).