Accumulation of LDL in Rat Arteries Is Associated With Activation of Tumor Necrosis Factor-α Expression

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Abstract—Activation of vascular inflammation in response to hyperlipidemia is believed to play an important role during the early stages of atherogenesis. We demonstrate here that exposure of cultured, rat aortic smooth muscle cells to low density lipoprotein (LDL) stimulated tumor necrosis factor-α (TNF-α) mRNA and protein expression. Oxidative modification of LDL resulted in a reduction of this stimulatory effect. To analyze whether a similar response also occurs in vivo, we used a recently developed model in which the effects of a rapid accumulation of human LDL in rat arteries can be studied. As previously reported, epitopes specific for human apolipoprotein B began to accumulate in the aorta within 2 to 6 hours after injection of 6 mg of human LDL. This was followed by expression of oxidized LDL-specific epitopes after 12 hours. There was no vascular expression of TNF-α at baseline or in phosphate-buffered saline–injected control rats. However, 24 hours after injection of native LDL, there was a marked induction of TNF-α mRNA and immunoreactivity in the aorta and other large arteries, whereas injection of oxidized LDL was without effect in this respect. Preincubation of LDL with the antioxidant probucol before injection markedly decreased the expression of TNF-α immunoreactivity. The present findings support the notion that LDL may activate arterial expression of TNF-α and suggest 1 possible mechanism for the inflammatory response in the early stages of atherosclerosis. The role of LDL oxidation in this process remains to be fully elucidated. (Arterioscler Thromb Vasc Biol. 2000;20:2205-2211.)

Key Words: atherosclerosis ■ oxidized LDL ■ probucol ■ TNF-α

The early stages of atherosclerosis involve increased retention of lipoproteins1-2 in the arterial wall, followed by activation of endothelial expression of leukocyte adhesion molecules, such as vascular cell adhesion molecule-1 and E-selectin,3-5 and penetration of mononuclear leukocytes through the endothelium into the intimal layer. Intimal monocytes differentiate into resident macrophages, which then ingest lipoprotein-derived cholesterol and develop into foam cells.6 The fact that macrophages have few receptors for normal LDL but a large capacity to ingest oxidized LDL by the scavenger receptor pathway has led to the conclusion that LDL entrapped in the arterial wall becomes oxidatively modified by reactive oxygen intermediates and/or enzymatically dependent processes.7,8 Oxidative modification of LDL is associated with the formation of proinflammatory substances such as lipid peroxides, platelet-activating factor–like phospholipids, and lysophosphatidylcholine,9 suggesting that lipid oxidation products may also be involved in activation of the inflammatory reaction in atherosclerosis.

The marked inflammatory component of the early atherosclerotic process has focused attention on the role of cytokines. Tumor necrosis factor (TNF)-α is of particular interest in this respect. It regulates endothelial expression of leukocyte adhesion molecules10,11 as well as endothelial procoagulant and fibrinolytic activity,12 activates the synthesis of growth factors and cytokines in vascular cells, and stimulates the growth of smooth muscle cells (SMCs).13 The presence of TNF-α has been demonstrated in human atherosclerotic plaques,14,15 in proliferating SMCs in the balloon-injured rabbit aorta,16 in balloon-injured rat femoral arteries,17 and in the media of coronary arteries during acute rejection of rabbit cardiac allografts.18 Moreover, circulating TNF-α levels are significantly increased in patients with premature coronary heart disease compared with age-matched healthy controls.19 TNF-α has also been implicated in the insulin resistance syndrome, a well-characterized risk factor for coronary heart disease.20,21 TNF-α is also expressed in adipose tissue and skeletal muscle and is believed to act locally by regulating the sensitivity of the insulin receptor.22,23 In these tissues, there is an association between increased lipid accumulation and TNF-α expression.24,25 We have recently developed an animal model to study the response of vascular tissue to a sudden and transient accumulation of lipoproteins in vivo.26,27 The aim of the present study was to determine whether exposure of vascular cells to LDL is associated with induction of TNF-α expression.

Methods

The following mouse monoclonal antibodies were used: anti-human apo B from Boehringer Mannheim; anti-rat monocyte and macro-
phage antibody (clone ED-1) obtained from Serotec; anti-rat TNF-\(\alpha\) supplied by R&D Systems; and anti-\(\alpha\)-actin (HHF-35) from Dako. Secondary antibodies were biotinylated horse anti-mouse IgG supplied by Vector and biotinylated sheep anti-\(\alpha\)-actin IgG from Pierce. The Limulus amebocyte lysate assay was performed by Microbiology Reference Laboratory (Cincinnati, Ohio). Male Sprague-Dawley rats were obtained from B&K Universal AB (Sollentuna, Sweden). Protocols was kindly provided by Dr Ann-Margret Lindquist, Astra-Hässle (Mölndal, Sweden). Lipopolysaccharide (LPS) was from Sigma, and PD-10 columns were from Pharmacia.

**Preparation and Oxidation of LDL**

Blood samples for LDL preparation were taken from healthy volunteers after 12 hours of fasting, during which time smokers were asked to refrain from smoking. All subjects were free from symptoms of infectious disease at the time of blood sampling. Venous blood was drawn into precooled Vacutainer (Becton-Dickinson) tubes containing Na\(_2\)EDTA (1.4 mg/mL) and placed on ice. Plasma was then recovered by low-speed centrifugation (1400 \(g\), 20 minutes, \(1^\circ\)C) and kept at this temperature throughout the preparation procedures. LDL was prepared by ultracentrifugation in a density gradient as described earlier.\(^2\) LDL was concentrated by pooling LDL prepared from at least 4 donors, adjusting the density to 1.065 g/L by adding NaBr containing 10 \(\mu\)mol/L CuSO\(_4\) for 18 hours at 37\(^\circ\)C. The LDL was then recovered by low-speed centrifugation (1400 \(g\), 20 minutes, \(1^\circ\)C). The LDL was frozen in 10% sucrose (vol/vol) at \(-80^\circ\)C. Cryopreserving the samples in sucrose did not affect the biological properties of LDL, as described previously.\(^3\) Within 1 hour before the injection of native LDL into the animals, excess salt and sucrose were removed by running the samples over a PD-10 column preequilibrated in PBS. The LDL was then sterilized by passing it through a 0.22-\(\mu\)m filter. The protein concentration of LDL was determined by the Limulus amebocyte lysate assay.

**Cell Culture**

SMCs were isolated from rat aorta as described previously.\(^4\) The cells were then grown in Ham’s F12/Dulbecco’s modified Eagle’s medium (GIBCO BRL) containing 10% newborn calf serum (GIBCO BRL), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin at 37\(^\circ\)C in an atmosphere of 5% CO\(_2\) in air. The purity of SMC populations was determined by the presence of smooth muscle-specific \(\alpha\)-actin immunoreactivity with HHF-35 antibody. SMCs used for analysis were seeded in 100-mm plates at 200 000 cells per dish. Subconfluent cultures were growth-arrested by incubation in serum-free Ham’s F12 medium supplemented with antibiotics for 48 hours. Experiments were performed on cells cultured for up to 10 passages.

**Animal Protocol**

Male Sprague-Dawley rats (400 to 500 g) were injected intraperitoneally with PBS, LPS, or human native or oxidized LDL while under ether anesthesia. The rats were killed by intracardiac injection of KCl at 0, 2, 6, 12, 24, or 48 hours while under anesthesia (30 mg ketamine and 3 mg/kg Rompun). Heparin (1000 IU/kg) was injected into the heart, followed by perfusion with 0.9% saline containing 0.02 mmol/L BHT for 5 minutes. Rats were perfusion-fixed for 10 minutes with 4% formaldehyde. Tissues were then placed in 4% formaldehyde for 6 to 12 hours and then transferred to a 15% sucrose solution and kept at 4\(^\circ\)C overnight.

**Immunohistochemistry**

Tissue sections were deparaffinized with xylene and dehydrated with graded ethanol. The membranes were permeabilized in 0.2% Triton X-100. Endogenous peroxidase activity was quenched by incubating the sections in 3% H\(_2\)O\(_2\), 80% methanol for 30 minutes at room temperature. After being washed, the sections were blocked with 10% horse serum in PBS for 30 minutes. Primary antibodies were diluted in PBS and incubated for 18 hours at 4\(^\circ\)C in a humidified chamber. The sections were washed and incubated with the biotinylated secondary antibody at a dilution of 1:200 for 30 minutes and then washed. The sections were incubated for 30 minutes with a peroxidase-labeled avidin-biotin complex and washed again. The sections were developed with diaminobenzidine (Vector) and counterstained in hematoxylin. Negative controls included substitution of the primary antibody with either PBS or an irrelevant antibody.

The TNF-\(\alpha\) immunoreactivity was graded by a blinded observer according to a modified version of the technique described by Galis et al.\(^6\) Consistent positive staining involving >50% of the vascular wall was recorded as (4); positive staining of 30% to 50% of the area as (3); positive staining of 5% to 30% of the area as (2); positive staining of 1% to 5% of the area as (1); and staining of <1% of the area as (0).

**RT-PCR Analysis of Rat TNF-\(\alpha\) mRNA**

For each group described above, 3 to 6 male Sprague-Dawley rats were studied to evaluate the presence of TNF-\(\alpha\) mRNA. Each animal was decapitated; their aorta, iliac, and femoral arteries were removed, rinsed in sterile saline, trimmed of adventitial tissue, and immediately stored in LN\(_2\). Other tissues were removed and treated similarly. RNA samples prepared from the livers of oxidized LDL-treated animals served as positive controls for the polymerase chain reaction (PCR) analysis (data not shown). Total RNA was purified by using RNAgent total RNA isolation system for reverse transcription (RT)–PCR (Promega), after which each RNA sample was treated with DNase (DNase I, Ambion) and repurified a second time. Two micrograms of total RNA was reverse-transcribed at 42\(^\circ\)C for 50 minutes by the random-priming method with Pd(N), hexamers (Pharmacia Biotech). Analysis of specific mRNAs by RT-PCR was accomplished by using a Cientech amplimer set. For our purpose, the rat TNF-\(\alpha\) amplifier set (5500-3) and rat \(\beta\)-actin amplifier set (5506-1) were used. An aliquot of each reaction was subjected to electrophoresis on 1.8% agarose gels. Bands were analyzed by ethidium bromide staining. Quantification of the TNF-\(\alpha\) and \(\beta\)-actin bands was performed with a Fuji Bas 1500 Biomaging analyzer. Results were expressed as the ratio of intensity of the TNF-\(\alpha\) to the \(\beta\)-actin bands.

**TNF-\(\alpha\) ELISA**

TNF-\(\alpha\) levels in culture medium and in rat serum were assayed by using high-sensitivity rat TNF-\(\alpha\) immunoassay plates (Amersham, Life Science).

**Statistical Methods**

Values are given as mean±SD. Between-group analyses were made with ANOVA followed by post hoc testing. A value of \(P<0.05\) was considered significant.

**Ethical Considerations**

This study was approved by the local institutional Animal Care and Use Committee.

**Results**

Expression of TNF-\(\alpha\) in Cultured Rat Aortic SMCs Exposed to LDL and Oxidized LDL

Cultured, serum-starved rat aortic SMCs did not contain detectable amounts of TNF-\(\alpha\) mRNA as assessed by RT-PCR. Exposure of SMCs to 50 \(\mu\)g/mL human LDL for 6 hours resulted in a marked expression of TNF-\(\alpha\) mRNA (Figure 1). Increasing the LDL level to 100 \(\mu\)g/mL did not result in any further stimulation of TNF-\(\alpha\) mRNA expression. Oxidative modification abolished most of the stimulatory effect of LDL on TNF-\(\alpha\) mRNA expression. No significant increase in the release of TNF-\(\alpha\) was found in SMCs exposed to up to 100 \(\mu\)g/mL LDL or oxidized LDL for 6 hours.
Incubation of SMCs with 50 μg/mL LDL for 12 hours resulted in a significant increase in the amount of TNF-α released from the cells (Figure 2). However, increasing the concentration of LDL, as well as oxidation of LDL, resulted in less activation of TNF-α release. Low concentrations of LDL and oxidized LDL (1 to 10 μg/mL) did not stimulate TNF-α mRNA and protein expression in the cells.

Vascular TNF-α Expression in Response to LDL

There was no vascular TNF-α immunoreactivity present in control rats (Figure 3A), whereas injection of 100 μg of LPS induced a marked expression of TNF-α in both arteries and veins (data not shown). We have previously shown that LDL accumulates in the rat vascular wall 6 to 24 hours after intravenous injection. This LDL accumulation is associated with modifications that lead to its recognition by antibodies generated against malondialdehyde- and hydroxynonenal-modified LDL.\textsuperscript{26} Injection of 6 mg of human LDL was found to activate vascular expression of TNF-α within 6 to 12 hours, depending on the size of the vessel. The earliest expression of TNF-α immunoreactivity was found in small arteries and veins (Figure 3B). At 6 hours, some TNF-α immunoreactivity was also present in the endothelial lining of larger arteries (Figure 3C). Twelve hours after administration of LDL, TNF-α immunoreactivity was present in the media of most arteries and showed a primarily cellular location (Figure 3D). At 24 hours, TNF-α immunoreactivity was abundant throughout the media in the aorta, iliac, and femoral arteries (Figure 3E). By later time points, the TNF-α staining had faded and completely disappeared 72 hours after injection of LDL (Figure 3F). A summary of the changes in expression of TNF-α immunoreactivity in the aorta, iliac, and femoral arteries after injection LDL is shown in Figure 4. SMCs in other organs did not express TNF-α after LDL injection. However, an increased expression of TNF-α was observed in the epithelium of the small intestine, adipose tissue, and the liver (data not shown). The levels of TNF-α in the sera of all animals were below the detection limit (50 pg/mL) of the rat TNF-α ELISA.

Effect of Injection of Oxidized LDL

Injection of human in vitro–oxidized LDL did not induce vascular TNF-α expression. Earlier studies had shown that preoxidized LDL is immediately removed by the liver and does not accumulate in the vascular wall. An increased expression of TNF-α was found in the liver 12 and 24 hours after injection of oxidized LDL (data not shown).

Effect of Probucol on Vascular TNF-α Expression in Response to LDL

To analyze the possible association between LDL oxidation in the vascular wall and activation of TNF-α expression, plasma was incubated with 50 μmol/L of the antioxidant...
probucol before isolation of LDL. This treatment resulted in a 4-fold increase in the lag phase for diene formation in response to copper (data not shown). Injection of probucol-loaded LDL resulted in the vascular accumulation of apo B comparable with that observed in animals given LDL, but the accumulation of malondialdehyde and hydroxynonenal antigens (ie, epitopes specific for oxidized LDL) was markedly reduced.26 Moreover, pretreatment with probucol resulted in almost complete inhibition of LDL-induced activation of vascular TNF-\(\alpha\) expression (Figure 5). This effect appeared

**Figure 3.** Vascular expression of TNF-\(\alpha\) after injection of human LDL. Positive staining was visualized with peroxidase-labeled avidin-biotin complexes (reddish-brown staining). A, Aorta from a PBS-treated control rat; B, expression of TNF-\(\alpha\) in small veins and arteries 6 hours after LDL injection; C, TNF-\(\alpha\) expression in aortic endothelium 6 hours after LDL injection; D, cell-associated expression of TNF-\(\alpha\) in femoral medial cells 12 hours after LDL injection; E, abundant accumulation of TNF-\(\alpha\) throughout the iliac media 24 hours after LDL injection; and F, absence of TNF-\(\alpha\) immunoreactivity in the aorta 72 hours after LDL injection. Original magnification \(\times\)200.

**Figure 4.** Time course of TNF-\(\alpha\) expression after LDL injection. The TNF-\(\alpha\) immunoreactivity was graded by a blinded observer according to a modified version of the technique described by Galis et al.26 Consistent positive staining involving >50% of the vascular wall was recorded as (4), positive staining of 30% to 50% of the area as (3), positive staining of 5% to 30% of the area as (2), positive staining of 1% to 5% of the area as (1), and staining of <1% of the area as (0). Each value represents the mean of 3 animals.
Activity of Vascular TNF-α mRNA Expression in Response to LDL and Oxidized LDL

To determine whether the vascular expression of TNF-α in response to LDL was associated with a local activation of TNF-α gene transcription, mRNA expression in the aorta and iliac arteries was analyzed by the RT-PCR technique. Equal loading was assessed by using primers specific for rat β-actin. All arteries in animals given LDL were found to contain TNF-α mRNA, whereas no expression of TNF-α mRNA was found in animals given PBS alone or probucol-loaded LDL (Figure 5).

One possibility is that the decrease in vascular TNF-α expression in rats given probucol-pretreated LDL is due to a direct inhibitory effect of probucol on SMC TNF-α expression. To investigate this possibility, interleukin-1–treated, cultured, rat SMCs were grown with or without 50 μmol/L probucol for 24 hours, and TNF-α mRNA levels were analyzed by RT-PCR. Interleukin-1 treatment resulted in a 3-fold increase in TNF-α mRNA levels in the cells, but this increase was not affected by addition of probucol (data not shown). To examine the possibility that the effect of LDL was mediated by factors generated during a cell-mediated oxidation process but not present in copper-oxidized LDL, cultured SMCs were exposed to LDL with or without 50 mmol/L probucol for 24 hours. Again, probucol was without effect on the induction of TNF-α mRNA expression (data not shown).

To further analyze whether the induction of TNF-α mRNA expression by LDL was caused by minor oxidative modifications by iron ions present in Ham’s F12 medium, SMCs were exposed to LDL in iron-free Eagle’s minimum essential medium. However, culture of the cells in this medium for 24 hours resulted in cell damage and activation of TNF-α mRNA expression that was even greater than that induced by LDL (data not shown).

Discussion

Activation of vascular inflammation in response to lipid accumulation is generally believed to play a critical role in the initiation of atherosclerosis. However, the mechanisms by which lipids activate the inflammatory response remain to be fully elucidated. Recent studies have shown that the cytokine TNF-α has important functions in the regulation of lipid and glucose metabolism. It is expressed in skeletal17 and adipose22–25 tissue and induces lipolysis through inhibition of the insulin receptor.23–38 It has been proposed that TNF-α acts as a “sensor” of fat cell lipid content and protects against lipid overload.39 If TNF-α has similar functions in the vascular wall, it represents a possible mechanism by which accumulation of lipids may initiate early inflammatory lesions.

The present study has demonstrated increased expression of TNF-α in cultured, aortic SMCs grown in the presence of LDL. Accumulation of LDL in the vascular wall in vivo also resulted in the activation of TNF-α expression. The results show that whereas no TNF-α is expressed in control rat arteries, abundant TNF-α immunoreactivity is induced after injection of human LDL. In the proximal part of the aorta, this immunoreactivity was predominantly located in the subendothelial layer, whereas in the distal aorta, iliac, and femoral arteries and in small, muscular arteries, TNF-α immunoreactivity was encountered throughout the medial layer. Generally, TNF-α staining was stronger in smaller than larger arteries.

Although the endotoxin level of LDL preparations used in the present study was <2 ng/mg LDL protein, the possibility that the effect of LDL on vascular TNF-α expression was due to LDL endotoxin contamination should be considered. However, preincubation of LDL with 100 μg/mL LPS diminished LDL-induced TNF-α expression and was even less potent than LPS alone (data not shown).

An increased expression of TNF-α in response to lipid accumulation could play an important role during the early phases of atherosclerosis. TNF-α is a potent activator of endothelial adhesion molecule expression.39 TNF-α is also known to activate SMC migration17 and proliferation.40 It is expressed in migrating SMCs after balloon injury of rat and rabbit arteries,17 by proliferating SMCs in the coronary arteries of rabbit rejected cardiac allografts,18 and in SMCs in human atherosclerotic plaques.14 Accordingly, induction of SMC production of TNF-α by LDL may also be involved in the activation of SMCs during the formation of fibromuscular lesions.

Several studies have suggested that oxidation of LDL is involved in the formation of early atherosclerotic lesions. Oxidized LDL stimulates endothelial adhesion molecule expression, acts as a chemottractant for monocytes, and stimulates the synthesis of other monocyte chemotactants, such as monocyte chemotactant protein-1. Treatment with antioxidants has also been shown to reduce plaque formation in hypercholesterolemic animals. Previous experiments in the same animal model used in this study have shown that the LDL that has accumulated in the arterial wall undergoes oxidative modification within 6 to 12 hours and that this change is accompanied by increased expression of adhesion molecules. Treatment with the antioxidant probucol partially inhibited the formation of oxidation products and expression of adhesion molecules but did not affect the extent of apo B...
Oxidized LDL has also been shown to activate expression of TNF-α in adherent monocytes. The present study suggests a complex role for the induction of TNF-α by oxidized LDL. Oxidized LDL injected into rats accumulates in the liver but not in the arteries. This situation is associated with marked expression of TNF-α in the liver. Injection of native LDL results in the activation of vascular TNF-α expression at the same time points at which oxidation of LDL occurs in the vessel wall. Pretreatment of LDL with probucol markedly reduced vascular TNF-α expression in response to LDL injection. In contrast to these findings, oxidized LDL failed to induce TNF-α expression in cultured SMCs. The observation that probucol failed to inhibit TNF-α synthesis induced by interleukin-1 in cultured SMCs suggests that the in vivo effect of probucol was not explained by a direct inhibition of TNF-α secretion unrelated to LDL oxidation. In accordance, Ku et al found that probucol lacked direct inhibition of TNF-α expression. In support of the latter possibility, activation of the proinflammatory transcription factor nuclear factor-κB in vascular cells by VLDL has been shown to involve the release of fatty acids and to be independent of oxidation. Another possibility that should be taken into account is the involvement of immune reactions. Previous studies have shown that injection of human LDL into rats initiates the production of antibodies, some of which form immune complexes with LDL. However, because vascular expression of TNF-α was already evident a few hours after LDL administration in the present study, this possibility is less likely. The mechanisms responsible for the increased expression of TNF-α in the intestinal epithelium, adipose tissue, and the liver in response to LDL injection remain to be clarified. However, it is interesting to note that all of these tissues have important functions in the regulation of lipid metabolism.

In summary, the present findings demonstrate that accumulation of LDL in the vascular wall is associated with an activation of vascular TNF-α expression, which could be involved in the formation of early inflammatory lesions. This induction may be due to LDL oxidation or an enzymatic release of phospholipids and fatty acids from aggregated LDL particles.

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