Toll-Like Receptor-4 Is Expressed by Macrophages in Murine and Human Lipid-Rich Atherosclerotic Plaques and Upregulated by Oxidized LDL

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Background—Inflammation is implicated in atherogenesis and plaque disruption. Toll-like receptor 2 (TLR-2) and TLR-4, a human homologue of drosophila Toll, play an important role in the innate and inflammatory signaling responses to microbial agents. To investigate a potential role of these receptors in atherosclerosis, we assessed the expression of TLR-2 and TLR-4 in murine and human atherosclerotic plaques.

Methods and Results—Aortic root lesions of high-fat diet–fed apoE-deficient mice (n=5) and human coronary atherosclerotic plaques (n=9) obtained at autopsy were examined for TLR-4 and TLR-2 expression by immunohistochemistry. Aortic atherosclerotic lesions in all apoE-deficient mice expressed TLR-4, whereas aortic tissue obtained from control C57BL/6J mice showed no TLR-4 expression. All 5 lipid-rich human plaques expressed TLR-4, whereas the 4 fibrous plaques and 4 normal human arteries showed no or minimal expression. Serial sections and double immunostaining showed TLR-4 colocalizing with macrophages both in murine atherosclerotic lesions and at the shoulder region of human coronary artery plaques. In contrast to TLR-4, none of the plaques expressed TLR-2. Furthermore, basal TLR-4 mRNA expression by human monocyte-derived macrophages was upregulated by ox-LDL in vitro.

Conclusions—Our study demonstrates that TLR-4 is preferentially expressed by macrophages in murine and human lipid-rich atherosclerotic lesions, where it may play a role to enhance and sustain the innate immune and inflammatory responses. Moreover, upregulation of TLR-4 in macrophages by oxidized LDL suggests that TLR-4 may provide a potential pathophysiological link between lipids and infection/inflammation and atherosclerosis. (Circulation. 2001; 104:3103-3108.)

Key Words: receptors ■ inflammation ■ cells ■ atherosclerosis ■ lipoproteins

Experimental work over the past decade has linked inflammation to atherogenesis and plaque disruption.1–4 The precise triggers for inflammation are not known but may include modified lipoproteins and local or distant infections.2 A potential role for infection in the development of atherosclerosis has been considered for several decades, but interest in this topic has recently reemerged because of several recent observations. Accumulating evidence has implicated specific infectious agents, including Chlamydia pneumoniae, in the progression and/or destabilization of atherosclerosis.1–17

Recent studies suggest that chlamydia lipopolysaccharide (LPS) induces foam-cell formation, whereas its heat-shock protein (chlamydia HSP60) induces oxidative modification of LDL.5,18 Chlamydia HSP60 has been implicated in the induction of deleterious immune responses in human chlamydial infection and has been found to colocalize with infiltrating macrophages in the atheroma lesions.19 Collectively, these data support a potential role for C pneumoniae in the development and progression of atherosclerosis and suggest that this organism may indeed play an active role in atheroma development. Available data, however, also underscore the current lack of a complete understanding of the molecular mechanisms that link C pneumoniae infection to innate immunity and trigger the signals for enhanced inflammation and atherogenesis.

LPS, a major component of the outer surface of Gram-negative bacteria, activates the proinflammatory transcription factor nuclear factor (NF)-κB in endothelial cells and macrophages.20,21 Recently, human Toll-like receptor-4 (TLR-4), a human homologue of drosophila Toll, has been identified as
the signaling receptor for endotoxin as well as human and chlamydial HSP60.

Currently, more than 10 human TLRs have been identified, and at least 10 human homologues of drosophila Toll have been sequenced. Whereas TLR-4 is used by enteric Gram-negative bacteria and LPS, TLR-2 is used by Gram-positive bacterial, mycobacterial, fungal, and spirochetal cell-wall components. TLRs are evolutionarily conserved innate immune receptors that recognize pathogen-associated molecular patterns and contain a common intracytoplasmic domain that conveys signals by molecules that are shared by interleukin-1 receptor signaling to activate the NF-κB pathway and release inflammatory cytokines. Because TLR-2 and TLR-4 play an important role in the innate immune and inflammatory responses, we investigated the expression of these receptors in murine aortic and human coronary atherosclerotic plaques. Here, we report preferential expression of TLR-4 in lipid-rich and macrophage-infiltrated murine and human atherosclerotic plaques. In vitro studies demonstrated basal expression of TLR-4 by macrophages, which was upregulated by oxidized LDL (ox-LDL). These findings suggest a potential role for TLR-4 in lipid-mediated proinflammatory signaling in atherosclerosis. Because TLR-4 is the receptor that recognizes chlamydial antigens such as chlamydia LPS and HSP60, it may provide a potential molecular link between chronic infection, inflammation, and atherosclerosis.

Methods
Preparation of Mouse Tissue
Apolipoprotein E (apoE)-deficient mice (C57BL/6J strain, 5 weeks old, 18 to 20 g) obtained from Jackson Laboratory (Bar Harbor, Me) (n = 5) were fed a high-fat, high-cholesterol (atherogenic) diet containing 42% (wt/wt) fat and 0.15% cholesterol from 6 weeks of age through the duration of the experiment. After anesthesia with efflurane, the mice were euthanized at 26 weeks of age, and their hearts and proximal aortas were excised and embedded in OCT compound (Tissue-Tek), frozen on dry ice, and then stored at −70°C until sectioning. Serial sections 10 μm thick were collected on slides for immunohistochemistry as described earlier.

Preparation of Human Tissue and Human Monocyte-Derived Macrophages
Human coronary artery specimens from 9 autopsy cases were collected within 24 hours of death, fixed with 10% formalin overnight, and embedded in paraffin. Five of the 9 coronary artery specimens included lipid-rich plaques containing a well-defined lipid core covered by a fibrous cap, and the other 4 of the 9 specimens included fibrous plaques, which contained mostly extracellular matrix without a lipid core. Normal mammary artery specimens were also obtained from 4 additional autopsy cases. Sections 5 μm thick were cut and applied to slides for both hematoxylin-eosin and immunohistochemical staining. Peripheral-blood monocytes were isolated from whole blood of normal human subjects by Ficoll-Paque density gradient centrifugation. Monocyte-derived macrophages were cultured in RPMI 1640 containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B for 5 days as described earlier.

Immunohistochemistry
Frozen sections of the apoE-deficient mouse aortic root were fixed with acetone for 5 minutes at room temperature and then immunostained with rabbit anti–hTLR-4 immune serum (1:100, obtained from Dr Ruslan Medzhitov, Yale University) according to the instructions on Dako’s immunostaining kit. Rat anti–mouse macrophage antibodies (1:500, Serotec) were used as macrophage marker. Colors were developed with the Dako AES substrate system. Smooth muscle cells were stained by a mouse anti-actin antibody conjugated with alkaline phosphatase (1:50, Sigma). Colors were developed with a Vector Red Alkaline Phosphatase Substrate Kit I. Rabbit IgG or rabbit serum was used as a negative control.

For human atherosclerotic plaques, after deparaffinization in graded alcohol, sections were immunostained with rabbit anti–human TLR-4 and TLR-2 antisera (1:100) raised against extracellular peptide domains of TLR-4 and TLR-2 as previously described. Preincubation of the anti–TLR-4 antisera with TLR-4 peptide (FKEIRHKLTRNFDNLNVKMT) was used to demonstrate specificity of the stain, and rabbit IgG or rabbit serum instead of primary antibody was used as negative control.

Double Immunohistochemistry
Double immunostaining of human atherosclerotic plaques was performed with Dako’s Doublestain System Kit. After TLR-4 immunostaining, 3,3′-diaminobenzidine was used as the peroxidase chromogenic substrate. Mouse monoclonal anti–human CD68 antibody (360 μg/mL, 1:20 dilution; Dako, Derma) for macrophages and mouse monoclonal anti–human α-actin antibody (100 μg/mL, 1:100 dilution; Dako, Derma) for smooth muscle cells were used with fast red as the alkaline phosphatase chromogenic substrate.

Preparation and Modification of Lipoproteins
Human native LDL (Sigma) was dialyzed against isotonic PBS (pH 7.4) to remove EDTA by use of Slide-A-Lyzer cassette 10 000 MWCO (Pierce). Ox-LDL was prepared as described previously. In brief, oxidation of LDL was performed by incubating 0.1 mg of LDL protein/mL with 5 μmol/L CuSO4 for 24 hours at 37°C. All reagents were endotoxin-free. LPS levels of LDL preparations were confirmed with a chromogenic Limulus assay and contained <0.3 pg of LPS/μg LDL protein. The extent of oxidation of the lipoprotein preparations was determined by the thiobarbituric acid-reactive substance (TBARS) assay. The ox-LDL had 20 to 25 nmol/L TBARS/mg cholesterol.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was isolated from resting and native LDL–, ox-LDL–stimulated human monocyte–derived macrophages with an RNA Stat60 isolation reagent (Tel-test “B” Inc) according to the manufacturer’s instructions and treated with RNase-free DNase I. For the reverse transcription (RT) reaction, the MMLV preamplification system (Life Technologies, Inc) was applied. Polymerase chain reaction (PCR) amplification was performed with Taq gold polymerase (Perkin Elmer) for 32 cycles at 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 minute (for TLR-2 and TLR-4). The oligonucleotide primers used for RT-PCR were TLR-2, 5'-GCCAAAGTCTTGATTGATTGG and 5'-TTGAAGTTCTC-GAGTCACC; for TLR-4, 5'-TGGATACGTGTCTCCTTAAAG and 5'-GAAATGGAGGCCACCTTCTC-5' as described earlier. GAPDH primers were obtained from Clontech.

Results
TLR-4 Is Expressed in Atherosclerotic Lesions of ApoE-Deficient Mice
In all 5 apoE-deficient mice, TLR-4 immunoreactivity was observed in the aortic root atherosclerotic lesions, which colocalized with macrophage immunoreactivity (Figure 1). TLR-4 staining was absent in the normal vessels obtained from control C56BL/6J mice. Mouse IgG staining was negative, and preincubation of the tissue sections with the specific peptide against which the anti–TLR-4 antisera was generated completely blocked the TLR-4 staining in the apoE-deficient vessels, indicating the specific nature of the peptide.
TLR-4 immunostaining. No TLR-2 immunoreactivity was observed (data not shown) in normal or atherosclerotic lesions.

**TLR-4 Is Expressed in Human Coronary Plaques**

The human coronary atherosclerotic plaques were classified into lipid-rich plaques containing a well-defined lipid core covered by a fibrous cap (n=5) and fibrous plaques that contained mostly extracellular matrix without a lipid core (n=4). Strong TLR-4 expression (brown staining) was observed around the lipid core and at the shoulder of lipid-rich plaques, where it colocalized with macrophage immunoreactivity (Figure 2). Incubation of the antiserum with the peptide used to generate the primary antibody blocked TLR-4 immunoreactivity, confirming the specificity of the anti–TLR-4 antiserum. Double staining showed close spatial colocalization of TLR-4 expression with macrophage immunoreactivity (Figure 2). No TLR-4 immunoreactivity or macrophage immunoreactivity was found in fibrous plaques that demonstrated strong smooth muscle α-actin immunoreactivity (Figure 2). Normal mammary arteries showed only minimal or no TLR-4 expression (Figure 2). TLR-2 immunoreactivity was absent in all plaques, whereas control staining was positive in THP-1 cells (data not shown).

**TLR-4 mRNA Regulation by Ox-LDL**

Cultured human monocyte-derived macrophages were stimulated with native LDL or ox-LDL for 5 hours, RT-PCR for TLR-2 and TLR-4 was performed, and relative intensity was calculated by densitometry as described earlier.32 RT-PCR showed basal TLR-2 and TLR-4 mRNA expression by macrophages. The TLR-4 mRNA was upregulated by ox-LDL in a dose-dependent manner up to 3-fold, whereas native LDL had no effect. TLR-2 mRNA was not upregulated by ox-LDL (Figure 3).

**Discussion**

Although precise triggers for inflammation in atherosclerosis are not fully understood, hypercholesterolemia, modified lipoproteins, and infection with organisms such as *C pneumoniae* and others have all been implicated. There is now evidence that *C pneumoniae* infection can accelerate the

**Figure 1.** TLR-4 immunoreactivity is seen within atherosclerotic plaque, in lipid core of plaque of aortic sinus of apoE-deficient mouse (A). B and C, Immunoreactivity of macrophages and smooth muscle cells, respectively, in serial section of aortic sinus. Note close spatial localization of macrophage immunoreactivity and TLR-4 immunoreactivity. D, Rabbit IgG staining for negative control. E, There is no immunoreactivity of TLR-4 in nonatherosclerotic aortic sinus of C57BL/6J mouse.

**Figure 2.** Photomicrographs showing immunohistochemical evidence for TLR-4 expression in human atherosclerotic lipid-rich plaques but not in fibrous plaques. A, Atherosclerotic plaque stained with rabbit anti–human TLR-4 antiserum (brown staining); B, negative control where primary antibody was replaced by rabbit IgG; C, TLR-4 immunoreactivity (brown); D, double immunostain with TLR-4 brown and macrophages red to demonstrate colocalization. E, F, and G, Higher-magnification view of macrophage immunoreactivity (red), TLR-4 immunoreactivity (brown), and macrophage plus TLR-4 immunoreactivity (red and brown), respectively. Fibrous plaques showed no immunoreactivity for TLR-4 (H) or macrophages (J) but showed only smooth muscle cell α-actin immunoreactivity (red) without TLR-4 immunoreactivity (brown) on double staining in I. K, Negative control with preabsorption of antiserum with peptide; L, normal mammary artery showing only minimal immunoreactivity to TLR-4 along endothelial border.
progression and facilitate the induction of atherosclerosis in cholesterol-fed rabbits and genetically modified atherosclerosis-prone mice. The concept of C pneumoniae–induced atherogenesis is further strengthened by the finding that antibiotic therapy against chlamydia prevents acceleration of atherosclerosis in the rabbit model. Ingalls et al have suggested LPS, and Kol et al have implicated HSP60, as the triggers for chlamydia-induced inflammatory responses. Both chlamydia infection and its LPS have been shown to induce foam-cell formation in monocytes. Persistence of LPS and/or HSP-60 in the atheroma either within intact C pneumoniae–infected cells or in the subendothelial space after cell lysis may promote atherosclerosis by continued macrophage activation. Indeed, circulating chlamydial LPS–specific immune complexes have been detected in patients with coronary heart disease. To date, however, the precise molecular mechanisms by which infections such as C pneumoniae contribute to the progression of atherosclerosis and the links among lipids, microbial antigens, and innate immune and inflammatory responses are not well understood.

Activation of monocytes/macrophages is an important initial step in the cascade of events leading to many inflammatory diseases, including atherosclerosis. The recent findings that TLR-4 is the signaling LPS receptor and also recognizes HSP60 provided a new impetus in elucidating the role of TLR-4 in various inflammatory diseases. Furthermore, a recent study showed that saturated fatty acids, but not unsaturated fatty acids, induce NF-κB activation and expression of cyclooxygenase-2 through the TLR-4 receptor as well.

In this study, we show for the first time that the proinflammatory signaling receptor TLR-4 is expressed in lipid-rich, macrophage-infiltrated atherosclerotic lesions of mice and humans and that TLR-4 mRNA in cultured macrophages is upregulated by ox-LDL but not by native LDL, whereas neither native LDL nor ox-LDL regulated TLR-2 mRNA.
might contribute to enhanced and chronic inflammation by signaling through the TLR-4 receptor, which is upregulated by ox-LDL.

Our findings of increased expression of TLR-4 induced by ox-LDL suggest a potential mechanism for the synergistic effects of hypercholesterolemia and infection in acceleration of atherosclerosis observed in experimental models\textsuperscript{35,36} and human epidemiological observations.\textsuperscript{99} Thus, these findings provide additional new insights into the link among lipids, infection/inflammation, and atherosclerosis.

In summary, we observed that human TLR-4 but not TLR-2 is expressed in murine and human lipid-rich atherosclerotic plaques, including areas infiltrated by macrophages. Furthermore, we show that ox-LDL but not native LDL induces upregulation of TLR-4 expression in macrophages. Given that TLR-4 plays a critical role in inflammatory and immune signaling, upregulated TLR-4 may participate in the inflammatory responses linking lipids to chronic infection, inflammation, and atherosclerosis. Improved understanding of the molecular mechanisms driving TLR-4 overexpression and signaling and the role of the resulting chronic inflammation during atherosclerosis may provide new targets for antiatherogenic therapy.

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