Expression of Toll-Like Receptors in Human Atherosclerotic Lesions: A Possible Pathway for Plaque Activation

Kristina Edfeldt, MSc; Jesper Swedenborg, MD, PhD; Göran K. Hansson, MD, PhD; Zhong-qun Yan, MD, PhD

**Background**—Innate immune reactions against bacteria and viruses have been implicated in the pathogenesis of atherosclerosis. To explore the molecular mechanism by which microbe recognition occurs in the artery wall, we characterized the expression of toll-like receptors (TLRs), a family of pathogen pattern recognition receptors, in atherosclerotic lesions.

**Methods and Results**—Semiquantitative polymerase chain reaction and immunohistochemical analysis demonstrated that of 9 TLRs, the expression of TLR1, TLR2, and TLR4 was markedly enhanced in human atherosclerotic plaques. A considerable proportion of TLR-expressing cells were also activated, as shown by the nuclear translocation of nuclear factor-κB.

**Conclusion**—Our findings illustrate a repertoire of TLRs associated with inflammatory activation in human atherosclerotic lesions, and they encourage further exploration of innate immunity in the pathogenesis of atherosclerosis. (*Circulation*. 2002;105:1158-1161.)

Key Words: receptors • atherosclerosis • nuclear factor-κB • immune system

Microorganisms such as *Chlamydia pneumoniae*, cytomegalovirus, and *Helicobacter pylori* are found in atherosclerotic lesions and may aggravate atherosclerosis in experimental models. Although infections do not seem to cause atherosclerosis, certain microbes might be pathogenetically important by aggravating and/or activating lesions. However, the mechanisms by which microbe recognition occurs in the artery wall have been unclear.

A family of toll-like receptors (TLRs) has recently been defined as a key component of pathogen-associated molecular pattern recognition machinery. Currently, at least 9 human TLRs have been identified. Ligation of these receptors initiates the activation of nuclear factor-κB (NF-κB), resulting in the expression of a wide array of inflammatory genes. A variety of bacterial and fungal components are known TLR ligands, including peptidoglycan for TLR2, lipopolysaccharide for TLR4, flagellin for TLR5, and unmethylated CpG motifs in bacterial DNA for TLR9. It is plausible that TLRs may collectively be responsible for sensing a large repertoire of microbial pathogens.

Frantz et al recently found that cardiac myocytes constitutively express TLR4 and that this expression is upregulated in the hearts of humans with cardiomyopathies and in rodents with experimental cardiac dysfunction. Furthermore, cultured vascular endothelial cells express TLR4 under baseline conditions, and they express high levels of TLR2 and TLR4 on stimulation with proinflammatory cytokines.

In the present report, we show that the expression of TLRs, in particular TLR1, TLR2, and TLR4, is markedly augmented in human atherosclerotic lesions; this augmentation occurs preferentially by endothelial cells and macrophages. A significant proportion of these cells were activated, as shown by the nuclear translocation of NF-κB.

**Methods**

**Specimen Collection**
Atherosclerotic plaques representing type VI lesions were obtained from patients undergoing carotid endarterectomy. Biopsies of the internal mammary artery were obtained from patients undergoing coronary bypass surgery. All samples were obtained with the informed consent of the patients after permission was obtained by the local ethics committee. In total, 5 normal arteries and 18 carotid plaques were examined in the present study.

**Semiquantitative Polymerase Chain Reaction**
Total RNA was isolated from the tissue samples using RNeasy Total RNA Mini Kit (Qiagen) with an additional phenol/chloroform extraction step and treated with RNase free DNase I (Qiagen). The RNA was then reverse-transcribed to cDNA using hexanucleotides and Superscript II reverse transcriptase (Life Technologies). The gene-specific primers were designed to amplify unique sequences in the 5’ parts of expressed TLR sequences. The speci-
ficity of selected primers for each TLR was confirmed using the Amplify 1.2 program for Macintosh. Sequences of TLR primers are available on request.

mRNA levels were assessed by semiquantitative polymerase chain reaction (PCR) in a reaction mixture containing 2 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 0.625 U Taq DNA polymerase, and 5 μCi ³²P-dATP/PCR reaction (NEN, DuMedical Scandinavia). PCR reactions were started at 95°C, and subsequent amplification was performed by denaturing for 40 s at 94°C, annealing (for 40 s at 56°C for TLR4; 62°C for TLR7, TLR8, and TLR9; and 60°C for other TLRs), and elongation for 60 s at 72°C. A total of 19 cycles were selected for β₂-microglobulin, 29 cycles for TLR9, and 25 cycles for other TLRs. The PCR products were separated on a 5% polyacrylamide gel and quantified by phosphoimaging. Expression of TLR mRNA was normalized to the β₂-microglobulin mRNA level for the same sample. PCR was also performed on samples without prior reverse transcription, and in no case was any DNA contamination present. For each sample, PCR reactions were performed twice, and the mean value was used for statistical analysis. The Mann-Whitney nonparametric test was used for comparisons.

Immunohistochemistry

Acetone-fixed cryostat sections were preincubated for 30 minutes in 5% normal serum and 2% dry milk powder. For staining TLR4 and TLR5, sections were first incubated for 30 minutes with mouse monoclonal anti-human CD64 (1:10 dilution, DAKO) to eliminate unspecific binding to Fc receptors. After incubation with primary antibodies at 4°C overnight, sections were incubated with biotinylated goat anti-rabbit (1:200) or horse anti-goat IgG (1:800), followed by avidin-biotin peroxidase complex (Vector Laboratories), and developed with diaminobenzidine. The specificity of TLR antibodies was assessed by incubating with control IgG from nonimmunized animals or by neutralizing the primary antibody with a 20-fold excess of blocking peptide at 4°C overnight.

For double-staining, TLRs were first stained as mentioned above. Thereafter, the sections were incubated overnight with antibodies to different cell markers and the p65 subunit of NF-κB, followed by incubation for 30 minutes with rabbit anti-mouse IgG and alkaline phosphatase anti-alkaline phosphatase (APAAP), and developed with FastBlue (Vector Laboratories).

The following antibodies were used in the present study: goat anti-human TLR1, goat anti-human TLR2, goat anti-human TLR3, rabbit anti-human TLR4, rabbit anti-human TLR5, goat anti-human TLR6 (all from Santa Cruz Biotechnology), mouse anti-human von Willebrand factor, anti-human CD68, anti-human α-smooth muscle actin (all from DAKO), anti-human CD3 (Becton Dickinson), and rabbit anti-human p65 (Santa Cruz Biotechnology).

Results

Semiquantitative reverse-transcriptase PCR was used to detect the expression of TLR mRNA in human arteries. Normal arteries showed very low levels of all TLRs, except for a relatively higher level of TLR4 mRNA (Figure 1). In atherosclerotic lesions, the abundance of TLR gene transcripts was considerably increased. In particular, TLR1, TLR2, and

![Figure 1. Enhanced expression of TLR genes in atherosclerotic lesions. TLR mRNA in internal mammary arteries (n=3; open boxes) and atherosclerotic plaques of carotid arteries (n=7; hatched boxes) was determined by semiquantitative reverse-transcriptase PCR and presented as the ratio between TLR and β₂-microglobulin mRNA. *P<0.05.](http://circ.ahajournals.org/doi/10.1161/01.CIR.105.23.1662)
TLR4 transcripts were elevated >3-fold compared with the level in normal arteries (Figure 1).

Immunohistochemistry showed the presence of TLR proteins with low staining intensity in the endothelium but not in deeper layers of the normal artery wall (Figure 2A). In contrast, TLR1, TLR2, and TLR4 were detected at high staining intensities in atherosclerotic plaques, especially in the endothelium and in areas infiltrated with inflammatory cells. A few cells within the plaque were positive for TLR5, and weak signals were registered for TLR3 and TLR6 (Figure 2A). Microheterogeneity in TLR expression due to variations in cellular composition might account for the variability in mRNA levels, despite the presence of TLR1, TLR2, TLR4, and TLR5 proteins in all lesions.

Double staining for TLRs and cell-type specific markers (Figure 2B) indicated that TLR2 and TLR4 were primarily expressed by CD68-positive macrophages and von Willebrand factor–positive endothelial cells. Occasional CD3-positive T cells also expressed TLR2 and TLR4 (data not shown). In contrast, few α-smooth muscle actin–positive smooth muscle cells showed staining for TLR2 or TLR4. TLR expression was associated with cellular activation, as indicated by the colocalized NF-κB p65 with TLR2 and TLR4 in a majority of endothelial cells and macrophages in all the lesions (Figure 2B).

**Discussion**

In the present study, we identified a repertoire of TLRs in human atherosclerotic lesions, as characterized by the upregulated expression of several TLRs in macrophages and endothelial cells of atherosclerotic lesions.

Although TLR2 and TLR4 were expressed at low levels by endothelial cells in normal arteries, the intensity of TLR staining was increased substantially in the endothelium covering atherosclerotic lesions. In these cells, both TLR2 and TLR4 frequently colocalized with NF-κB, a transcription factor that induces TLR expression but also mediates the downstream signaling of TLR on ligand engagement. Similarly, the presence of NF-κB p65 was also observed in TLR-positive macrophages. This suggests that the TLR–NF-κB pathway is activated in the lesion. However, the spatial correlation between TLR and p65 does not per se
prove that TLR ligation caused NF-κB activation. It will therefore be important to identify TLR ligands in lesions and to test this hypothesis in functional studies.

TLRs play a key role in the innate immune response. Our present data suggest that they may also be involved in atherosclerosis. Clinical studies have shown that infections with several pathogens, including *Chlamydia pneumoniae*, are associated with cardiovascular disease.6 *C pneumoniae* may signal through TLR4 to induce the proliferation of human vascular smooth muscle cells.7 Because neither infection nor the expression of TLR4 is sufficient to induce atherosclerosis in animal models,8,9 it is unlikely that microbes and/or TLR signaling play a causative role in this disease. Instead, they may be important as activators of silent disease. For example, microbial components such as lipopolysaccharide or lipoteichoic acid released during acute infection or exacerbation of a chronic infection might activate plaque cells. It has been proposed that such local “echos” of systemic infection could lead to heightened local production of cytokines and initiate plaque activation and rupture.10 The present finding of TLR expression in plaques immediately suggests a pathway through which such an echo effect could take place.

The range of upregulated TLRs in atherosclerotic lesions implies that TLR-binding ligands may not be limited to TLR4 binding lipopolysaccharide and TLR2 binding components of the Gram-positive cell wall. Cooperation between different TLRs may recognize other ligands, thus expanding the repertoire of the innate immune recognition complex.11,12 Finally, it cannot be ruled out that, like scavenger receptors, TLRs may also recognize products of eukaryotic cells under certain conditions. This possibility obviously requires further studies.

TLR expression was largely confined to endothelial cells and macrophages, suggesting that these 2 cell types are the primary responders to microbial challenge in the plaque. TLR expression by macrophages permits local differentiation of these cells into antigen-presenting cells because TLR2 ligation stimulates this process.13 Therefore, TLRs may provide a link between local innate and adaptive immunity and enhance the cellular immune response in the plaque to local antigens such as heat shock proteins and oxidized lipoproteins.

In conclusion, our data show that TLRs are expressed in atherosclerotic plaques and associated with inflammatory activation of endothelial cells and macrophages. This suggests a mechanism by which microbes may cause inflammatory plaque activation. We speculate that the TLR–NF-κB pathway is important for plaque destabilization in conditions such as acute coronary syndromes. This possibility will be tested in future studies.

**Note Added in Proof**
After the submission of this manuscript, Xu et al showed that TLR4 is expressed in murine and human lesions.14

**Acknowledgments**
This work was supported by the Swedish Heart-Lung Foundation and the Swedish Medical Research Council (Project Nos. 14079 and 6816).

**References**
Expression of Toll-Like Receptors in Human Atherosclerotic Lesions: A Possible Pathway for Plaque Activation
Kristina Edfeldt, Jesper Swedenborg, Göran K. Hansson and Zhong-qun Yan

Circulation. 2002;105:1158-1161
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/10/1158

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at: http://circ.ahajournals.org//subscriptions/