In Vivo Evidence for a Role of Toll-Like Receptor 4 in the Development of Intimal Lesions

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Background—Inflammation plays an important role in atherogenesis. The toll-like receptor 4 (TLR4) is the receptor for bacterial lipopolysaccharides and also recognizes cellular fibronec tin and heat shock protein 60, endogenous peptides that are produced in response to tissue injury. To explore a possible role for this receptor in arteri al obstructive disease, we determined the expression of TLR4 in the atherosclerotic arterial wall, including adventitia, and studied the effect of adventitial TLR4 activation on neointima formation in a mouse model.

Methods and Results—Localization of TLR4 was studied in human atherosclerotic coronary arteries by immunohistochemistry and detected in plaque and adventitia. In the adventitia, not all TLR4-positive cells colocalized with macrophages. In primary human adventitial fibroblasts, expression of TLR4 was demonstrated by immunofluorescence, Western blot, and reverse transcriptase–polymerase chain reaction. Adding lipopolysaccharide to these fibroblasts induced activation of NF-κB and an increase of mRNAs of various cytokines. The effect of adventitial stimulation of TLR4 was studied in a mouse model. A peri-adventitial cuff was placed around the femoral artery. Application of lipopolysaccharide between cuff and artery augmented neointima formation induced by the cuff (intimal area, 9134±1714 versus 2353±1076 µm², P<0.01). In TLR4-defective mice, application of cuff and lipopolysaccharide resulted in a smaller neointima than in wild-type mice (intimal area, 3859±904 µm², P=0.02 versus wild type).

Conclusions—A functional TLR4 is expressed in human adventitial fibroblasts and macrophages. Adventitial TLR4 activation augmented neointima formation in a mouse model. These results provide evidence for a link between the immune receptor TLR4 and intimal lesion formation.

Key Words: atherosclerosis • inflammation • adventitia • fibroblast • immune system

Atherosclerosis is considered an inflammatory disease. The presence of inflammatory cells in the intimal layer can be observed in all stages of atherosclerosis. A number of factors in the serum and endothelium, such as the high concentration of lipoproteins and endothelial dysfunction, probably play an important role in the influx of bone marrow–derived cells from the lumen into the arterial wall. The influx of macrophages and T-lymphocytes in atherosclerosis is not limited to the luminal side of the arterial wall. Adventitial inflammation has been associated with clinically manifested atherosclerotic lesions. The composition of these adventitial inflammatory infiltrates suggests a local immune response, but the trigger for this immune reaction is unknown. Infections have been suggested as potential triggers of arterial inflammation, but results have been conflicting and no causal relationship has been demonstrated.

In the innate immune system, cells can be activated by pathogens via the recently characterized family of toll-like receptors. The toll-like receptor 4 (TLR4) is specific for lipopolysaccharide (LPS), which is a major component of the outer layer of Gram-negative bacteria. In addition, cellular fibronec tin, which is produced in response to tissue injury, and heat shock protein 60 (HSP60), which can be either autologous or from microorganisms, have also been described as activators of TLR4. The expression of TLR4 has recently been described in macrophages and endothelial cells in the atherosclerotic plaque. However, in vivo evidence for involvement of this receptor in the development of intimal lesions has not yet been provided.

We studied the location of cells expressing TLR4 in the human atherosclerotic arterial wall and observed expression in parts of the plaque but also in a large number of cells in the adventitia.
adventitia. In the adventitia, fibroblasts are the most prominent cells and the involvement of adventitial fibroblasts in the formation of intimal lesions has been demonstrated in animal models.\textsuperscript{12,13} We report that next to macrophages, human adventitial fibroblasts express a functional TLR4. Activation of TLR4 in adventitial fibroblasts induced activation of NF-κB and the production of cytokines, suggesting that this cell type has TLR4-dependent immunoregulatory functions. To determine the role of TLR4 in the development of intimal lesions, we used a mouse femoral cuff model. Adventitial stimulation of TLR4 augmented neointima formation, an effect that was reduced in TLR4-defective mice. This result provides evidence for a link between the immune receptor TLR4 and intimal lesion formation.

**Methods**

**Immunohistochemistry**

Human coronary artery segments (n=5) were obtained post mortem. Frozen sections were fixed in acetone containing 0.15% H\textsubscript{2}O\textsubscript{2} and blocked with 10% normal goat serum. Sections were incubated with rabbit antihuman TLR4 immune serum (Vector Laboratories, Burlingame, Calif) containing 1% normal human serum, followed by streptavidin-horseradish peroxidase incubation and treatment with sodium acetate buffer containing 3-amino-9-ethyl carbazole. As negative control, the serum was replaced with PBS. For double staining of macrophages and TLR4, macrophages were visualized by detecting acid phosphatase, as described previously,\textsuperscript{14} and TLR4 was visualized with alkaline phosphatase (blue).

Mouse femoral artery paraffin sections were blocked with 10% normal horse serum and incubated with mouse α-actin (10 μg/mL, Sigma) for 1 hour at room temperature. Next, sections were incubated with biotin-labeled horse antimouse (Vector Laboratories, Burlingame, Calif) and visualized as above.

**Primary Human Adventitial Fibroblasts**

A small part of the thoracic aorta was dissected from human donor and recipient hearts (n=5) during heart transplantation. The adventitia (layer that was dissected (stripped) from the aorta and cells were isolated with the use of collagenase. The cells that attached in the first 6 hours were cultured in DMEM (Invitrogen), supplemented with L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL, and 10% fetal bovine serum (Invitrogen), and used throughout passages 2 to 4. Extraction of RNA and protein was performed with the use of Tripure Isolation Reagent (Roche). Cells were activated with 100 ng/mL LPS (Escherichia coli serotype 055.B5, Sigma).

**Immunocytochemistry**

For double-immunofluorescence staining of TLR4 and vimentin in human primary fibroblasts, cells were fixed with formaldehyde and blocked with 10% goat milk in PBS/0.1% Tween 20. Blots were incubated with rabbit antihuman TLR4 immune serum (1:1500) and biotinylated goat antirabbit antibody, and, finally, incubation with streptavidin-fluorescein (Vector).

**Western Blotting**

Denatured protein samples (8 μg/lane) were separated on an 8% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amer- sham Pharmacia). Incubation steps were done in 5% defatted dry milk in PBS/0.1% Tween 20. Blots were incubated with rabbit antihuman TLR4 immune serum, biotinylated goat antirabbit antibody, and, streptavidin-horseradish peroxidase, respectively, and visualized with the use of the chemiluminescence technique.

**Reverse Transcriptase–Polymerase Chain Reaction**

Isolated RNA (n=5) was treated with DNase and subsequently tested for the presence of genomic DNA by polymerase chain reaction without reverse transcription. Total RNA was converted to cDNA by use of the Ready to Go You Prime First system (Amer- sham). A primer set specific for TLR4 (5′-TCAGCCT-TGCTTCTAAGACTAC-3′ and 5′-ACACCACAAACTACCTTTC-3′) was used for amplification during 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. The identity of the amplified TLR4 cDNA was confirmed by sequence analysis.

**RNA Protection Assay**

RNA (2 μg) was used in an RNAse protection assay (Pharmingen). Radioactive RNA probes were generated with the use of templates HCK-2 and HCK-5 and T7 RNA polymerase (Roche). Protected fragments were quantified and compared with the housekeeping gene GAPDH after film development by use of Gel Doc 1000 system (BioRad).

**Electrophoretic Mobility Shift Assay**

To isolate nuclei, cells were lysed in buffer containing 10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl\textsubscript{2}, 10 mmol/L KCl, 0.1% Nonidet P-40, 0.2 mmol/L PMSF, and 0.5 mmol/L DTT. After centrifuging, protein was isolated by suspending the nuclei in buffer containing 20 mmol/L HEPES-KOH, 1.5 mmol/L MgCl\textsubscript{2}, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L DTT, and 0.2 mmol/L PMSF on ice and centrifuged. Binding reactions were performed with 10 μg nuclear protein and 0.1 pmol 32P-labeled oligodeoxynucleotide in buffer containing 4% glycerol, 1 mmol/L MgCl\textsubscript{2}, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.5, 0.05 mg/mL poly(dI-dC) (Amersham) in the absence or presence of anti-p65 NF-κB antibody (Santa Cruz Biotechnologies, Santa Cruz, Calif). DNA complexes were separated on a 4% polyacrylamide gel. The oligodeoxynucleotides contained the NF-κB or AP-1 binding site (Promega, Leiden, Netherlands).

**Animal Experiments**

The local ethics committee on animal experiments approved all animal experiments. Female wild-type BALB/c and C3H-Tlr4\textsuperscript{−/−} mice (Jackson Laboratory, Bar Harbor, Me), aged 12 to 20 weeks, were anesthetized with the use of 0.025 mL/10 g body weight of a cocktail (ketaminehydrochloride 115, 3 mg/mL, xylazine 10 mg/mL, atropine 0.5 mg/mL, NaCl 0.9% in the ratio 7:6:2:7, respectively). A nonconstrictive polyethylene cuff (0.40-mm inner diameter, 0.80-mm outer diameter, length 2.0 mm; Portex) was placed loosely around the right femoral artery.\textsuperscript{16,17} Between the artery and the cuff, 2% gelatin with or without LPS (1 μg/mL) was injected (BALB/c mice with LPS, n=9; BALB/c mice without LPS, n=9; C3H-Tlr4\textsuperscript{−/−} mice with LPS, n=8). At 3 weeks, mice were perfused with PBS containing 0.1mg/mL nitroglycerin and subsequently with 4% formaldehyde. Paraffin sections of the right femoral artery were stained with elastin–Van Gieson and hematoxylin-eosin. Three equally spaced cross sections were used in each mouse. With the use of computerized morphometric analysis, the intimal cross-sectional area between the lumen and the internal elastic lamina and the medial area between the internal and external elastic lamina were measured. The Mann-Whitney test was used to compare groups. Values are presented as mean±SEM.

**Results**

**TLR4 Is Expressed in Adventitial Cells**

We determined the expression of TLR4 in the arterial wall of human atherosclerotic coronary arteries with immunohistochemistry. We observed immunostaining in areas in the atherosclerotic plaque and in a large number of cells in the adventitia (Figure 1a). We then studied which adventitial cells express TLR4. Expression of TLR4 has predominantly...
been described in inflammatory cells. Because it is known that the adventitia of human atherosclerotic arteries contains macrophages, we performed a double staining for TLR4 and macrophages. In both adventitia and plaque we observed colocalization (Figure 1b). Not all cells with TLR4 immunostaining double-stained for macrophages (acid phosphatase), suggesting that other adventitial cells also express TLR4 (Figure 1b).

**TLR4 Is Expressed in Adventitial Fibroblasts**

Fibroblasts are abundantly present in the adventitia. We hypothesized that next to the adventitial macrophage, the adventitial fibroblast also expresses TLR4. To determine whether TLR4 is expressed in adventitial fibroblasts, we isolated primary adventitial fibroblasts from the adventitia stripped of human aortas. Double-immunofluorescence staining for TLR4 and vimentin demonstrated the expression of TLR4 in these vimentin-positive cells (Figure 2, a and b). Expression of TLR4 was confirmed by Western blot (Figure 2c) and reverse transcriptase–polymerase chain reaction (data not shown).

**Increased NF-κB Activity Via TLR4**

Activation of TLR4 induces activation of the transcription factor NF-κB. To examine the effect of TLR4 activation on NF-κB activity in adventitial fibroblasts, we added LPS to primary cultures of human adventitial fibroblasts for 1 hour. Nuclear extracts of these cells were subjected to electrophoretic mobility shift assay (EMSA) and showed a shift specific for NF-κB (Figure 3). Thus, adding LPS to human adventitial fibroblasts resulted in activation of NF-κB.

**Induction of Cytokine Production Via TLR4**

Signaling via TLR4 leads to the activation of NF-κB, which induces the transcription of proinflammatory genes. We studied whether activation of primary human adventitial fibroblasts via TLR4 resulted in an increase of mRNA levels of various cytokines. Therefore, we cultured primary human adventitial fibroblasts in the presence of LPS for 0, 6, 12, and 24 hours. With the use of an RNase protection assay we measured the mRNA levels of different cytokines. We observed an upregulation on mRNA level of the following cytokines: IL-1α (24× increased), IL-1β (42×), IL-1Ra (20×), IL-6 (14×), IFN-γ (16×), RANTES (110×), interferon-inducible protein-10 (IP-10, 130×), macrophage inflammatory protein (MIP-1β, >50×), monocyte chemoattractant protein-1 (MCP-1, 100×), and IL-8 (50×) (Figure 4). For all cytokines, the highest mRNA level was observed at 6 or 12 hours. IL-12p35, IL-12p40, Ltn, MIP-1α, and I-309 were not detected.

**Adventitial TLR4 Activation in Wild-Type and TLR4-Defective Mice**

Having demonstrated that adventitial cells express TLR4 and produce proinflammatory cytokines after LPS stimulation, we hypothesized that activation of TLR4 via the adventitial application of LPS results in migration of cells to the intima to form a neointima. To test this hypothesis, we studied the effect of periadventitial TLR4 activation on neointima for-
mation by placing a nonconstrictive cuff around the femoral artery of BALB/c mice and injecting gelatin with or without LPS between the cuff and the artery. After 3 weeks' follow-up, the group with adventitial LPS application showed a larger intimal area compared with the group without LPS (intimal areas 9134±1714 and 2353±1076 μm², respectively, P<0.01, Figure 5). Thus, adventitial LPS application augmented neointima formation induced by a cuff. C.C3H-Tlr4<sup>def/d</sup> mice are BALB/c mice that express a nonfunctional TLR4. To study whether the LPS-induced neointima formation was induced via TLR4, we performed the same operation in this mouse strain. In the C.C3H-Tlr4<sup>def/d</sup> mice, cuff placement and LPS-gelatin application resulted in a smaller neointima formation (3859±904 μm²) compared with the wild-type BALB/c mice treated with cuff and LPS-gelatin (P=0.02, Figure 5).

**Discussion**

Inflammatory and immune reactions probably play an important role in the development of atherosclerosis. The inflammatory reaction in the atherosclerotic arterial wall is not limited to the luminal side of the vessel wall. Inflammatory infiltrates are frequently present in the adventitia of atherosclerotic arteries, and the abundant expression of human histocompatibility leukocyte antigen-DM in the adventitia suggests an active adventitial inflammatory process. Infectious organisms and autologous structures, such as HSP60, have been suggested as triggers of an arterial inflammatory reaction. Recently, the TLR4 has been suggested as a link between infection/inflammation and atherosclerosis. Thus far, 3 structures have been described as activators of TLR4. First, TLR4 recognizes LPS, a part of the outer membrane of Gram-negative bacteria. Recent studies demonstrated that particles of the Gram-negative bacterium *Chlamydia pneumoniae* are frequently present in the adventitia of atherosclerotic arteries. Second, HSP60 has been described to activate the TLR4. Atherosclerosis has been associated with HSP60, which could be autologous or originate from microbial organisms. Third, TLR4 recognizes an alternatively spliced fragment of cellular fibronectin, which is produced in response to tissue injury. In vivo experiments demonstrated that this fragment was present in the adventitia of injured arteries. The TLR4 might, therefore, serve as a receptor by which both bacterial particles and arterial injury can induce an inflammatory reaction.

Two recent studies described expression of TLR4 in cells of the atherosclerotic plaque. These studies, however, were descriptive and focused on the atherosclerotic plaque, whereas the adventitia remained unexplored. The principal findings of the present study are that adventitial cells, including adventitial fibroblasts, express a functional TLR4 in human atherosclerotic arteries, and that the activation of TLR4 by adventitial application of LPS leads to the augmentation of intimal hyperplasia, which is considered the “soil” of atherosclerosis.

Having demonstrated that not all TLR4-positive cells in the adventitia are macrophages, we searched to find which other cell type expresses this receptor. Recent insights have led to the concept that fibroblasts are key sites of cytokine synthesis, which initiates a cascade of events leading to inflammation. We demonstrated that adventitial fibroblasts, as the only vimentin-positive cell present in the adventitia, can act as immunologic cells and that signaling through TLR4 on adventitial fibroblasts leads to the production of proinflammatory cytokines. LPS activated NF-κB in human primary adventitial fibroblasts. This result is in accordance with the observation that a constitutively active mutant of TLR4 can...
induce the activation of NF-κB and with the finding that LPS induces NF-κB activity in cells that were transiently transfected with human TLR4. Activation of NF-κB leads to the synthesis of a number of proinflammatory mediators. We show that LPS induces the synthesis of a range of cytokines in human adventitial fibroblasts. Particularly, the chemokines RANTES, IP-10, MIP-1β, MCP-1, and IL-8 were highly upregulated after LPS exposure. All these chemokines are chemoattractants of monocytes and T cells and have been described in association with atherosclerotic disease. Proliferation and migration of smooth muscle cells (SMCs) are considered an important event in the development of atherosclerosis. A number of the cytokines that we observed to be upregulated in adventitial fibroblasts after TLR4 activation (IP-10, IL-1, IL-6, IL-8) influence smooth muscle proliferation and/or migration. In experiments with mice, cytokines like IL-1 and IL-6 have been directly associated with the development of atherosclerosis. Because there are no specific markers to distinguish fibroblasts from SMCs and other undifferentiated cells, the layer of origin from the cells in the intima remains unclear. Migration of SMCs can be stimulated via cytokines produced by adventitial fibroblasts, and the migration of fibroblasts can be stimulated after TLR4 activation with the use of LPS, suggesting that both cell types can contribute to intima formation after TLR4 activation.

We used a well-described mouse model of cuff placement around the adventitia of the femoral artery. In this model, in which the induction of intima hyperplasia is minimally triggered by cuff placement alone, local activation of TLR4 by LPS in the adventitia induced an augmentation of the intima consisting of α-actin–positive cells. In the mouse strain with a defective TLR4, LPS gelatin application resulted in a smaller neointima than in the wild-type strain, which indicates that TLR4 was involved in neointima formation after adventitial LPS activation. A previous study showed that the implantation of an LPS-soaked cotton thread in the adventitia of the rat femoral artery induced intimal formation. We show that TLR4 is involved in this process and adventitial fibroblasts can act as immune-regulatory cells.

In summary, TLR4 is expressed in human adventitial fibroblasts and macrophages. Activation of TLR4 in human adventitial fibroblasts induces the production of proinflammatory cytokines, suggesting that adventitial fibroblasts are able to act as immune-regulatory cells. Activation of TLR4 by LPS in the adventitia augmented neointima formation in a mouse model, and the use of TLR4-defective mice confirmed involvement of TLR4 in this process. These results for the first time link the immune receptor TLR4 to intimal lesion formation in an in vivo situation. The TLR4 might be considered a potential candidate in the search for new targets to intervene in the development of arterial obstructive diseases.

Acknowledgments

This study was supported by the Netherlands Organization for Scientific Research (grants 902-16-222 and 906-26-213) and the Dutch Heart Foundation (grants 99-209 and 2001-162). We thank Dr R. Medzhitov (Yale University School of Medicine, New Haven, Conn) for providing the antihuman TLR4 immune serum, L.A. Kleij and R.E. Verloop for their technical help, and the heart transplantation team of our hospital for collaborating in obtaining the primary adventitial fibroblasts.

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Circulation. 2002;106:1985-1990; originally published online September 16, 2002;
doi: 10.1161/01.CIR.0000032146.75113.EE
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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