The Antiviral Cytomegalovirus-Inducible Gene 5 Is Expressed in Atherosclerosis and Regulated by Proinflammatory Agents


Objective—Inflammatory processes play an important role in atherosclerosis, and increasing evidence implies that microbial pathogens and proinflammatory cytokines are involved in the development and activation of atherosclerotic lesions. To find new inflammatory genes, we explored the vascular transcriptional response to an activator of innate immunity bacterial lipopolysaccharides (LPSs).

Methods and Results—Gene arrays identified the cytomegalovirus-inducible gene 5 (cig5) among the genes most potently induced by LPS in human vascular biopsies. Cig5 was expressed by endothelial cells in atherosclerotic arteries and significantly elevated in atherosclerotic compared with normal arteries. In culture, cytomegalovirus infection, interferon-γ, and LPS induced cig5 expression.

Conclusion—Cig5 is expressed in atherosclerosis and induced in vascular cells by inflammatory stimuli and cytomegalovirus infection. The putative functions of cig5 in atherosclerosis may relate to disease-associated microbes.

Key Words:

Development of atherosclerosis is associated with vascular inflammation. Recruited macrophages produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), whereas T cells activated by disease-associated antigens in lesions secrete interferon-γ (IFN-γ). The inflammatory milieu in the vessel exerts strong effects on the vascular endothelial and smooth muscle cells. They respond by expressing adhesion molecules, cytokines, and chemokines, which contribute to the atherosclerotic process. Clinical manifestations of atherosclerosis, such as myocardial infarction, appear to be triggered by this inflammatory process.

Although it is well established that atherosclerosis is an inflammatory disease, the initiating mechanisms are not fully understood. Several contributing factors have been proposed, including oxidized lipoproteins, bacteria, and viruses. Chlamydia pneumoniae and cytomegalovirus (CMV) have been detected in human atherosclerotic lesions, and epidemiological and animal studies suggest an association between these pathogens and disease. Microbial products ligating pattern recognition receptors may also contribute to plaque inflammation.

To investigate the vascular response to inflammation, we used gene arrays followed by single-gene studies. In this way, the CMV-inducible gene 5 (cig5), a virus-inducible antiviral protein, was identified as a putative culprit molecule in vascular inflammation and atherosclerosis.

Methods

The studies were approved by the ethical committee at the Karolinska Hospital and the ethics committee for animal experiments in Stockholm. Patients were included after informed consent.

Human Biopsies

A total of 53 patients scheduled for carotid endarterectomy and 13 patients scheduled for nephrectomy were included. Nine biopsies from the atherosclerotic carotid and 7 from the renal artery were incubated with or without lipopolysaccharide (LPS) from Escherichia coli, O55:B5 (Sigma Chemical) at 100 ng/mL for 6 hours. A portion of 3 of the renal artery biopsies was incubated with or without LPS for 24 hours. The incubated biopsies and unincubated 44 atherosclerotic and 6 renal artery biopsies were frozen. Serum was also obtained from 44 random reference patients free from medication and evidence of cardiovascular disease.

Animal Experiments

Fourteen apolipoprotein E–deficient (apoE−/−) mice were euthanized using CO₂ anesthesia at 20 weeks of age. The aorta was freed from...
connective tissue under a dissection microscope. An atherosclerotic segment from the ascending aorta and a macroscopically normal segment from the descending aorta from each vessel were frozen. From 7 apoE−/− and 7 C57BL/6 mice euthanized at 25 weeks of age, the aorta was frozen.

Cell Culture
Human aortic smooth muscle cells (Clonetics) and human coronary artery smooth muscle cells (CASMCs; Clonetics) were cultured in medium SmBM with SmGM2 (Clonetics). Human umbilical vein endothelial cells (HUVECs; Clonetics) were maintained in medium EBM-2 with EGM-2 (Clonetics). The following incubations were performed: medium only, 100 ng/mL LPS (Sigma), 10 ng/mL TNF-α, IL-1β, and IFN-γ (PeproTech), a mix of TNF-α, IL-1β, and IFN-γ (each 10 ng/mL; CM) infected at a multiplicity of infection between 0.1 and 1, with the endothelial-adapted CMV strain V1814 (a generous gift from Dr Giuseppe Gerna, University of Pavia, Italy), the laboratory strain AD169, Towne, or mock infection with supernatants from uninfected cells. Viral titers were determined as described previously.8

Real-Time RT-PCR
RNA was isolated using the RNeasy extraction kit including a deoxyribonuclease step (Qiagen), quality controlled using an Agilent Bioanalyzer 2100, and reverse-transcribed to cDNA. mRNA levels were assessed by real-time RT-PCR. Primers and probes for human TNF-α, IL-1β, cig5, and murine cig5 were designed using Primer-Express software (Applied Biosystems; sequences available on request) and for β-actin and cyclophilin as described. Monoycte chemoattractant protein-1 and IL-8 reagents were purchased from Applied Biosystems. Samples were analyzed using an ABI Prism 7700 Sequence Detector. Results were normalized to cyclophilin in the human and β-actin in the murine experiments.

Gene Array
RNA with a 28S/18S ratio >2.0 from 1 renal artery and from 8 pooled carotid lesions were analyzed using Affymetrix gene expression arrays U95A according to manufacturer instructions.

Enzyme-Linked Immunosorbent Assay
Serum from patients included in this study was tested for human CMV (HCMV)-specific IgG and IgM in an enzymost anti-HCMV/ IgG ELISA and an enzymost anti-HCMV/IgM ELISA (Behring).

Immunohistochemistry
Acetone-fixed cryostat sections were incubated with 2.5% normal horse serum for 30 minutes, followed by rabbit anti-cig5 polyclonal 1:3000 at 4°C overnight. Subsequently, the ImmPRESS Universal Antibody anti-rabbit Immunoglobulin Kit (Vector Laboratories) was used according to manufacturer instructions. Staining for von Willebrand factor was performed as described previously.7

Statistics
The Mann–Whitney U test was used and P<0.05 considered significant. Values are expressed as mean±SEM. For CMV prevalence, 2-group c2 tests were used.

Results
Upregulation of CMV induced genes on LPS stimulation of arterial tissue. The transcriptional response to LPS of a nonatherosclerotic human renal artery and pooled carotid atherosclerotic lesions were evaluated using Affymetrix global expression arrays. For the renal artery, 6828 of 12 558 (54%) transcripts gave a detectable signal. The expression of 276 transcripts increased and 258 decreased after LPS stimulation. For the atherosclerotic biopsies, 5580 of 12 558 (44%) transcripts were detected. The expression of 283 transcripts increased and 182 decreased after LPS stimulation. Cig5 and cig49 were among the 3 most strongly induced in the normal and atherosclerotic biopsies (Table).

Cig5 Expressed in Human Atherosclerotic Lesions and Upregulated by LPS in Human Arteries
Real-time RT-PCR showed 3.8-fold higher cig5 mRNA levels in human atherosclerotic lesions than in normal arteries (4.09±0.621 versus 1.06±0.199; P=0.012). After LPS stimulation, cig5 mRNA increased in renal arteries (19.8±5.14 versus 1.19±0.271; P=0.009) and carotid lesions (20.1±5.37 versus 2.42±0.357; P=0.006). The cig5 protein was expressed in human atherosclerosis but not in normal arteries (Figure 1) and induced by in vitro treatment of renal arteries with LPS (Figure 1). Immunohistochemistry revealed cig5 in the endothelium of human atherosclerotic lesions, which were of type V–VI, but not in nonatherosclerotic renal arteries (Figure 2). The prevalence of CMV infection was higher in patients (40 of 44; mean age 71.9 years) compared with a reference group (31 of 44; mean age 62.0 years; P=0.0012). No significant difference in cig5 expression was observed between anti-CMV IgG-positive and IgG-negative individuals in the patient group (P=0.49). No patients were positive for anti-CMV IgM.

Cig5 Expressed in Atherosclerotic Lesions of ApoE−/− Mice
RNA was isolated from atherosclerotic and nonatherosclerotic segments of 20-week-old apoE−/− mice. Lesions in apoE−/− mice of this age in our colony are usually advanced atheromata without evidence of plaque rupture. Cig5 mRNA was substantially higher in the atherosclerotic segments compared with nonatherosclerotic ones from the same group of mice (18.2±2.3 versus 4.5±0.83 arbitrary units; P<0.0001). A similar difference in cig5 expression was seen between whole aortas from 25-week-old apoE−/− mice compared with C57BL/6 mice (data not shown).

LPS, IFN-γ, and CMV Infection Induced Cig5 in HUVECs and Cerebrovascular Smooth Muscle Cells
Cig5 protein was induced in HUVECs and cerebrovascular smooth muscle cells (CVSMCs) in response to LPS, CMV, and IFN-γ but not in response to TNF-α or IL-1β (Figure 1). Both cell types also displayed strong mRNA induction in response to LPS, a cytokine mix (Figure 3a), or CMV (Figure 3b). No difference was observed between mock and uninfected cells. After addition of CMV to cultures, changes in cell morphology became visible in the light microscope after 72 to 96 hours (ie, ≈48 hours after cig5 induction [data not shown]).

Discussion
In the present report, we used gene array to explore the human vascular transcriptional response to an activator of innate immunity: LPS. The antiviral protein cig5 was identified as one of the most profoundly induced genes.
We subsequently detected cig5 expression in human and murine atherosclerosis. mRNA levels were significantly higher in lesions than in normal arteries, and the protein was detected in the endothelium of human atherosclerotic lesions but not in normal arteries. Cig5 expression could be induced by LPS, IFN-γ, and CMV in human endothelial and smooth muscle cells and was also induced by LPS in normal human arteries.

The strong induction of cig5 and cig49 in response to LPS in human vessels suggests that inflammatory stimulation may promote a similar response in the artery as CMV infection. Although cig49 is yet poorly characterized, cig5 is conserved between species and known to efficiently inhibit CMV infection when overexpressed in human fibroblasts. Cig5 contains a motif associated with protein radical formation and biosynthesis of cofactors, which may influence the antimicrobial defense. Hence, cig5 may be important in the local defense against CMV, a putative atherosclerosis-related pathogen.

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Numbers indicate fold induction compared with control.

Figure 1. Expression of 43-kDa cig5 analyzed by Western blot in cultured arteries with or without LPS stimulation for 24 hours (a), snap-frozen arterial biopsies (b), and cultured cells after 24 hours of stimulation with different cytokines or CMV infection (c). We subsequently detected cig5 expression in human and murine atherosclerosis. mRNA levels were significantly higher in lesions than in normal arteries, and the protein was detected in the endothelium of human atherosclerotic lesions but not in normal arteries. Cig5 expression could be induced by LPS, IFN-γ, and CMV in human endothelial and smooth muscle cells and was also induced by LPS in normal human arteries.

The strong induction of cig5 and cig49 in response to LPS in human vessels suggests that inflammatory stimulation may promote a similar response in the artery as CMV infection. Although cig49 is yet poorly characterized, cig5 is conserved between species and known to efficiently inhibit CMV infection when overexpressed in human fibroblasts. Cig5 contains a motif associated with protein radical formation and biosynthesis of cofactors, which may influence the antimicrobial defense. Hence, cig5 may be important in the local defense against CMV, a putative atherosclerosis-related pathogen.

Figure 2. Immunohistochemical staining for cig5 in an atherosclerotic lesion at ×50 (a) and ×400 (b) magnification and in renal artery at ×50 (c) and ×400 (d) magnification. The atherosclerotic lesion has a well-organized cap and endothelial cig5, whereas the renal artery displays intimal thickening but no atherosclerosis and no cig5.
In our experiments, cig5 expression could be induced by several different stimuli (ie, by LPS, CMV, and IFN-γ but not by TNF-α or IL-1β). Multiple ways of induction have also been demonstrated by others.12,13,16,17 The induction by different viruses and by LPS16,18 suggests an involvement of pathogen-associated molecular pattern receptors capable of initiating responses against evolutionarily distant stimuli.13 Interestingly, Fas-dependent cell death also promotes activation of an antiviral response. In this way, cig5 induction may synergize with cytotoxic attack in the vascular defense against CMV and other viral infections that may play a role in vascular disease. However, the induction pathway of cig5 is not known in detail. Because TNF-α and IL-1β did not induce cig5, it is not likely that nuclear factor κB activation alone upregulates cig5. Although human data are limited, given that multiple agents are capable of cig5 induction, most likely, cig5 is also induced during inflammatory processes other than CMV defense. In support of this, the cig5 levels in biopsies from atherosclerotic lesions did not correlate to CMV seropositivity. However, the prevalence of anti-CMV IgG was higher in individuals with symptomatic atherosclerosis than in a healthy reference group. Importantly, a primary CMV infection was not detected in any patients.

We detected cig5 in the endothelium of human atherosclerotic lesions but not in other cell types or in normal arteries. Cig5 induction after CMV infection was 100-fold stronger in HUVECs as in CASMCs (Figure 3a). We therefore conjecture that the pathways of induction can differ between cell types, with CMV infection being a strong activator in the endothelium. The detection of cig5 in atherosclerotic lesions may thus be linked to CMV but also to an active immune status in the lesion. Consequently, the role of cig5 in vascular inflammation needs to be further evaluated.

In conclusion, this study shows that cig5, an evolutionarily conserved antiviral component of the innate immune response, is expressed in atherosclerotic arteries and strongly induced in vascular cells by inflammatory stimuli and CMV infection. The putative functions of cig5 in atherosclerosis may relate to disease-associated microbes. Further studies are needed to determine its pathogenic importance.

Acknowledgments
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