Innate Immune Recognition of Invasive Bacteria Accelerates Atherosclerosis in Apolipoprotein E–Deficient Mice

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Background—Infectious diseases have emerged as potential risk factors for cardiovascular disease (CVD). Epidemiological studies support a connection between periodontal disease, a chronic inflammatory disease of the supporting tissues of the teeth, and CVD.

Methods and Results—To directly test the connection between periodontal disease and atherosclerosis, apoE−/− mice were orally challenged with the periodontal disease pathogen Porphyromonas gingivalis or an invasion-impaired P gingivalis fimbriae-deficient mutant (FimA−). Both wild-type P gingivalis and the FimA− mutant were detected in blood and aortic arch tissue of apoE−/− mice by PCR after challenge. ApoE−/− mice challenged with wild-type P gingivalis presented with increased atherosclerotic plaque and expressed the innate immune response markers Toll-like receptor (TLR)-2 and TLR-4 in aortic tissue. Despite detection of the FimA− mutant in the blood and in aortic arch tissue, apoE−/− mice challenged with the FimA− mutant did not present with periodontal disease, upregulation of TLRs, or accelerated atherosclerosis. Furthermore, we demonstrate that immunization to control P gingivalis–elicited periodontal disease concomitantly prevents P gingivalis–accelerated atherosclerosis.

Conclusions—We conclude that invasive P gingivalis accelerates atherosclerosis. (Circulation. 2004;109:2801-2806.)

Key Words: infection • inflammation • atherosclerosis • receptors • endothelium

Cardiovascular disease (CVD) is a common cause of death in the western world. Despite identification and study of factors that predispose humans to CVD, including diet, metabolism, exercise, and genetics, an incomplete picture of the pathogenesis of CVD has emerged because of the use of gene-targeted animals, such as the apolipoprotein E (apoE)–knockout (apoE−/−) mouse, that develop severe hyperlipidemia and accelerated atheroma formation. Inflammation is a determining factor for development of CVD, with emphasis on a mononuclear cellular infiltrate, the host response to oxidized LDL, cytokines, C-reactive protein levels, cell adhesion molecule expression, and identification of Toll-like receptor (TLR)-4 at the site of atherosclerotic plaque accumulation. TLRs are an emerging group of pattern recognition molecules that mediate the innate host response to microbes and are selectively upregulated after infection.

Chronic infectious diseases, including periodontal disease, are associated with increased risk for CVD. Periodontal disease is a chronic inflammatory disease of the periodontium that leads to erosion of the attachment apparatus and supporting bone for the teeth and is one of the most common chronic infectious diseases of humans. Porphyromonas gingivalis is the primary pathogenetic agent of adult periodontal disease. Haraszthy et al detected P gingivalis in human atheromatous tissue by polymerase chain reaction (PCR), indicating that P gingivalis gains access to the vasculature and localizes at sites of atheroma development. Additional studies also support that P gingivalis can aggravate CVD. P gingivalis possesses a broad array of virulence factors, including proteases, lipopolysaccharides, capsular polysaccharide, hemagglutinins, and fimbriae. In vitro studies demonstrate that the fimbriae of P gingivalis play a significant role in attachment and invasion of endothelial cells, stimulation of cell adhesion molecule production, and chemokine expression. Furthermore, a P gingivalis fimbria–deficient mutant failed to elicit oral bone loss in a rat oral infection model.

In this study, we demonstrate that only invasive P gingivalis accelerates atherosclerosis in apoE−/− mice with the
Detection and Quantification of *P. gingivalis* From Blood and Aortic Arch of ApoE⁻/⁻ Mice During Oral Challenge

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>PCR Detection of <em>P. gingivalis</em></th>
<th><em>P. gingivalis</em> in Sample (CFU/µg DNA)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Aortic Arch</td>
</tr>
<tr>
<td>None</td>
<td>0/8</td>
<td>0/7</td>
</tr>
<tr>
<td><em>P. gingivalis</em> WT</td>
<td>8/8</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FimA⁻ mutant</td>
<td>7/8</td>
<td>2/7</td>
</tr>
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*Two mice from each group were euthanized at 2 hours (for blood) or 24 hours (for aorta) after the 3rd, 6th, and 12th challenges and after the final challenge. The data are presented as the number of animals positive for *P. gingivalis* total No. of samples.†Total DNA was isolated and quantified from the blood and aortic arch of mice, and 16S PCR was performed using *P. gingivalis*-specific 16S-specific primers. DNA from a known number of viable *P. gingivalis* was serial 10-fold diluted and separated on agarose gels with test samples. Semiquantitative densitometric analysis was performed to determine the numbers of *P. gingivalis* present in each sample. The data are presented as the mean equivalent of *P. gingivalis* per µg isolated DNA±SD. The numbers in parentheses are the high and low for each group. ND indicates not detected.

upregulation of TLR-2 and TLR-4. Furthermore, immunization with *P. gingivalis* before oral challenge prevents *P. gingivalis*-accelerated atherosclerosis.

**Methods**

**Oral Challenge and Immunization**

*P. gingivalis* strain 381 and its fimbriae-deficient mutant DPG3 (FimA⁻) were grown as described. Five-week-old male apoE⁻/⁻ and C57BL-6 mice (Jackson Laboratories, Bar Harbor, Me) were cared for in accordance with Boston University Institutional Animal Care and Use Committee procedures and received a standard chow diet. Mice were challenged with *P. gingivalis* 381 or FimA⁻ in 2% carboxymethyl cellulose. Some groups of apoE⁻/⁻ mice were immunized subcutaneously 2 times per week for 3 weeks with heat-killed *P. gingivalis* 381 whole-organism preparations without adjuvant.

**16S PCR of *P. gingivalis* and RT-PCR**

**PCR of Blood**

Whole blood (100 µL) was collected from each mouse during the oral challenge regimen. Total DNA was collected using a QiaAmp kit (Qiagen), and the *P. gingivalis* 16S gene was detected by polymerase chain reaction (PCR).

**PCR and RT-PCR of Aortic Arch Tissue**

The aortic arch was harvested from apoE⁻/⁻ mice after saline perfusion, and the tissue was homogenized with a sterile, RNase-free tissue homogenizer. These samples were prepared for total RNA extraction using an RNasey column (Qiagen), and the fluid from the first column wash (DNA-enriched fraction) was collected and used for 16S PCR of *P. gingivalis*. Total RNA was then collected according to the manufacturer’s instructions and used for amplification of murine TLR-2, TLR-4, and β-actin.

**Measurement of Serum Levels of *P. gingivalis*-Specific IgG, Cholesterol, and Triglycerides**

Serum levels of *P. gingivalis*-specific IgG was determined by ELISA. Total cholesterol (Sigma) and triglycerides (Sigma) were determined according to the manufacturer’s instructions.

**Assessment of Periodontal Bone Loss**

Oral bone loss was determined at the maxillary molars of all mice at 17 weeks of age as described previously.

**Anti-Mouse TLR-2 Antibody**

The TLR-2 monoclonal antibody was generated by immunizing Lewis rats with Chinese hamster ovary/mouse TLR-2 cells and fusing the spleenocytes to NSO/1 mouse myeloma cells. A rat IgG2b,k antibody clone was chosen on the basis of recognition of Chinese hamster ovary/mouse TLR-2 and nonreactivity with Chinese hamster ovary/human TLR-2 or Chinese hamster ovary/human TLR-4 by fluorescence-activated cell sorting (FACS).

**Assessment of Atherosclerosis and Immunohistochemistry**

Animals were euthanized 6 weeks after challenge, at 17 weeks of age. The aorta of each mouse (n=10 mice per group) was harvested from the aortic valve to the iliac bifurcation, opened longitudinally, and stained with Sudan IV. Digital micrographs were taken of the aortic arch, and the total area of atherosclerotic plaque was determined from on-screen images using IPLabs (Scanalytics, Inc) by an observer blinded to the identity of the samples. A subset of animals were perfused with saline and 4% paraformaldehyde, and the aortic arch with heart tissue was harvested and embedded. Eight-micrometer cryosections were collected; probed with anti-mouse TLR-2, anti-human TLR-4, or isotype-matched antibodies; developed; and counterstained; and images were recorded using a digital camera attached to a light microscope.

**HAEC Cell Culture**

Confluent monolayers of human aortic endothelial cells (HAECs; Cascade Biologics) in 6-well plates were challenged with wild-type (WT) *P. gingivalis* or the FimA⁻ mutant at a multiplicity of infection of 100. Similar cultures were incubated with either high (10 µg/mL) or low (1 µg/mL) doses of *P. gingivalis* FimA protein isolated from *P. gingivalis* 381.

**Fluorescence-Activated Cell Sorting**

HAECs cultured for 2, 6, and 24 hours with *P. gingivalis* WT, the FimA⁻ mutant, or FimA protein were washed, fixed, and probed with FITC-labeled TLR-2, TLR-4, or isotype-matched antibodies (Biocarta), and FACS analysis was performed on 10,000 cells.

**Statistical Analysis**

The data are presented as the mean±SD. One-way ANOVA with Tukey-Kramer multiple-comparisons test was performed to assess differences in total atherosclerotic plaque accumulation, and a value of *P*<0.05 was considered significant.
Results

Bacteremia and Localization of \textit{P. gingivalis} in Aortic Arch Tissue of apoE\textsuperscript{-/-} Mice After Oral Infection

\textit{P. gingivalis} was detected in the blood of mice by 16S PCR throughout the challenge regimen, whereas the FimA\textsuperscript{-} mutant was detected only after the final oral challenge (Table). In addition, 16S PCR of aortic arch tissue at the site of predicted accelerated atheroma formation\cite{16,38,42,43} revealed that both WT and FimA\textsuperscript{-} mutant were present in these tissues (Table). Unchallenged C57BL-6 and apoE\textsuperscript{-/-} mice were negative for \textit{P. gingivalis} transcripts.

Serum Analysis and Oral Bone Loss

Unchallenged apoE\textsuperscript{-/-} mice possessed high cholesterol and triglyceride levels, and oral challenge with WT \textit{P. gingivalis} or the FimA\textsuperscript{-} mutant had no effect on the levels of these molecules (Figure 1A). WT and mutant \textit{P. gingivalis} stimulated similar levels of \textit{P. gingivalis}-specific IgG, suggesting no differences in the adaptive host response to either organism (Figure 1B). C57BL/6 and apoE\textsuperscript{-/-} mice developed oral bone loss to WT \textit{P. gingivalis}, whereas the FimA\textsuperscript{-} mutant failed to stimulate oral bone loss (data not shown).\cite{35}

\textit{P. gingivalis} Oral Infection Accelerates Atherosclerosis in ApoE\textsuperscript{-/-} Mice

Mice challenged with WT \textit{P. gingivalis} possessed significantly more atheroma on the intimal surface of the aortic arch compared with unchallenged animals (Figure 1, C and D). Mice challenged with the FimA\textsuperscript{-} mutant failed to accelerate atheroma, as the level of deposited plaque resembled unchallenged mice (Figure 1, E and F). We did not observe progression of atherosclerotic plaque into the thoracic or abdominal regions of the aorta.

Invasive \textit{P. gingivalis} Oral Infection Elicits TLR Expression in the Aortic Arch of ApoE\textsuperscript{-/-} Mice

RT-PCR revealed increased expression of TLR-2 and TLR-4 in aortic tissue of mice challenged with WT \textit{P. gingivalis}. Animals challenged with the FimA\textsuperscript{-} mutant were negative for TLR-2 and TLR-4 transcripts (Figure 2A). Using immunohistochemistry, we observed low levels of TLR-2 in aortic

Figure 1. Oral challenge of apoE\textsuperscript{-/-} mice with WT \textit{P. gingivalis} stimulates accelerated atherosclerotic plaque formation. A, Serum analysis (n=10 mice/group) of total cholesterol and triglyceride levels from unchallenged (black bars), WT \textit{P. gingivalis} (gray bars), or \textit{P. gingivalis} FimA\textsuperscript{-} mutant (hatched bars) challenged mice. B, Serum levels of \textit{P. gingivalis}-specific IgG before oral challenge (blue bars) or at 6 weeks after challenge (purple bars) with WT \textit{P. gingivalis} (WT) or FimA\textsuperscript{-} mutant (FimA\textsuperscript{-}). C, Unchallenged, (D) WT \textit{P. gingivalis}, and (E) FimA\textsuperscript{-} mutant are representative of atherosclerotic plaque present on intimal surface of aortic arches of mice. F, Morphometric analysis of total area of atherosclerotic plaque of unchallenged (None), WT \textit{P. gingivalis}, or FimA\textsuperscript{-} mutant challenged mice (FimA\textsuperscript{-}). *\textit{P}<0.05; NS indicates not significant vs unchallenged mice. Magnification \times40; bar=1 mm.

Figure 2. ApoE\textsuperscript{-/-} mice orally challenged with invasive \textit{P. gingivalis} express increased TLR-2 and TLR-4 in aortic arch tissue. A, RT-PCR amplification of TLR-2, and TLR-4 mRNA from aortic arch tissue of unchallenged (None), WT \textit{P. gingivalis} (WT), or mutant \textit{P. gingivalis} (FimA\textsuperscript{-}) challenged mice. B, Immunohistochemical confirmation of TLR-2 and TLR-4 expression in aortic tissue of mice. IRR-Ab indicates irrelevant isotype-matched antibody. Magnification \times100; bar=10 \textmu m.
tissues of unchallenged mice (Figure 2B), whereas elevated TLR-2 was observed in aortic tissue sections of mice challenged with WT *P gingivalis*. Slight TLR-2–specific staining was observed in tissue sections from mice challenged with the *P gingivalis* FimA/H11002 mutant. TLR-4 expression was observed only in the aortic sinus of mice challenged with invasive *P gingivalis* (Figure 2B).

**HAECs Infected With WT *P gingivalis* Express TLRs**

FACS analysis revealed surface expression of TLR-2 and TLR-4 on HAECs cultured with WT *P gingivalis* at 2 and 6 hours of coculture. By 24 hours after challenge, TLR expression returned to levels similar to those of unstimulated cells. The FimA– mutant failed to stimulate TLRs and resembled unstimulated cells (Figure 3A). HAECs cultured with purified FimA protein did not express TLRs and resembled unstimulated cells (Figure 3B). These results suggest that *P gingivalis* invasion, and not the FimA protein itself, was required for the upregulation of TLR expression.

**Immunization to Prevent *P gingivalis*–Elicited Periodontal Disease Ameliorates *P gingivalis*–Accelerated Atherosclerosis in ApoE<sup>-/-</sup> Mice**

Immunization of mice with heat-killed *P gingivalis* elicited a potent *P gingivalis*–specific IgG response and prevented *P gingivalis*–elicited oral bone loss (data not shown). Morphometric analysis of atherosclerotic plaque accumulation on the intimal surface of the aortic arch of mice revealed that immunization with heat-killed *P gingivalis* protected animals from *P gingivalis*–accelerated atherosclerotic plaque accumulation (Figure 4). These results demonstrate that immunization prevents both *P gingivalis*–mediated periodontal disease and *P gingivalis*–accelerated atherosclerosis.

**Discussion**

Conflicting reports exist regarding the ability of infectious diseases, such as periodontal disease, to aggravate CVD. Here, we report that oral challenge of apoE<sup>-/-</sup> mice, in a manner that elicits periodontal disease, accelerates atheroma formation. Studies using site-relevant animal model challenge regimens have demonstrated that *Chlamydia pneumoniae* infection can accelerate atherosclerosis. Previously, Hu et al reported that strain-dependent differences were important in the ability of *C pneumoniae* to accelerate atherosclerosis in LDL receptor–deficient mice placed on a high-fat diet. It was demonstrated by immunohistochemistry that aortic tissue harvested from mice that were infected with *C pneumoniae* strains AR39 or MoPn possessed *Chlamydia*-specific antigens; however, only strain AR39 stimulated accelerated atheroma development. Similar levels of IgG were ob-
served in each Chlamydia strain, suggesting that the adaptive immune response was not responsible for the observed differences in atheroma development. Until our present report, detailed studies that incorporate a genetically defined bacterial mutant to address the pathogenesis of infection-induced atherosclerosis have not been performed. We demonstrate that the mechanism by which P gingivalis adheres to and/or invades vascular tissue is critical to the stimulation of accelerated atheroma development, because a P gingivalis FimA− mutant failed to accelerate atherosclerosis despite evidence of bacteremia and localization of the mutant in the aorta. Although both the WT and the FimA− mutant can gain access into the vasculature and to the aorta, our data support the notion that the FimA− mutant fails to activate the endothelium.

TLRs are pattern recognition receptors of cells that sense the external environment, and it is reported that TLR-2 and TLR-4 play a role in the host response to P gingivalis lipopolysaccharides and fimbriae. However, the importance of the TLR-mediated response during P gingivalis-mediated periodontal disease is unknown. Studies using Mycobacterium avium and Haemophilus influenzae support the proposition that bacterial infection leads to increased expression of TLRs and that these TLRs play a role in further innate immune sensing during Crohn’s disease and tuberculosis. Our studies demonstrate that during P gingivalis–accelerated atherosclerosis, the host modulates the expression of TLRs, but only to invasive organisms. Furthermore, the upregulation of the innate immune response precedes accelerated atheroma development. The hypothesis that an upregulated innate immune response is associated with atherosclerosis is not new. What has emerged from our studies is that the mechanism by which an infectious agent adheres to or invades the host and the subsequent correlation of regulated TLR expression as part of the innate immune response to this infection are critical to the outcome of accelerated atherosclerosis. On the basis of our in vitro and in vivo data, we conclude that only fully invasive P gingivalis initiates accelerated plaque accumulation. We also infer that merely the localization of P gingivalis in the aortic tissue, capture of noninvasive P gingivalis by the spontaneously developing atheromatous plaque, or the presence of P gingivalis is insufficient to drive accelerated atheroma formation.

Although CVD is a multifactorial disease, using a combination approach consisting of a defined genetic mutant of P gingivalis, a site-specific challenge regimen, within an effective animal model for assessment of accelerated atherosclerosis, we demonstrate an experimental link between P gingivalis oral infection and exacerbation of atherosclerotic plaque accumulation. Most importantly, our data suggest that specific vaccination to prevent periodontal disease caused by P gingivalis concomitantly reduces the risk of infection-accelerated CVD.

Acknowledgments

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