Porphyromonas gingivalis Infection Accelerates the Progression of Atherosclerosis in a Heterozygous Apolipoprotein E–Deficient Murine Model

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Background—Current epidemiological data suggest that dental infections affecting tooth-supporting tissues (periodontitis) can disseminate into the systemic circulation and thereby contribute to atherosclerosis progression. To test this hypothesis, we investigated the effect of repeated systemic inoculations with Porphyromonas gingivalis (Pg), a putative periodontal pathogen, on the progression of atherosclerosis in heterozygous apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice.

Methods and Results—Ten-week-old, male ApoE<sup>−/−</sup> mice fed either a high-fat diet or regular chow were inoculated intravenously with live Pg (10<sup>7</sup> CFU) or vehicle once per week for 10, 14, or 24 consecutive weeks. Histomorphometry of plaque cross-sectional area in the proximal aortas, en face measurement of plaque area over the aortic trees, Pg 16S ribosomal DNA amplification with polymerase chain reaction, ELISA for systemic proinflammatory mediators, and immunolocalization of macrophages in the proximal aorta were performed. Atherosclerotic lesions of the proximal aortas and aortic trees were more advanced in Pg-challenged animals than in vehicle control animals and occurred earlier (at 10 weeks) when no lesions were apparent in control animals. At 24 weeks after inoculation, proximal aortic lesion size quantified by histomorphometry was 9-fold greater in Chow-fed mice inoculated with Pg than in noninoculated mice (<i>P</i>&lt;0.001) and was 2-fold greater in Pg-inoculated versus noninoculated high-fat diet–fed mice (<i>P</i>&lt;0.001); all atherosclerotic lesions were macrophage-rich. Pg ribosomal DNA was found in the aortas, livers, and hearts 24 weeks after inoculation.

Conclusions—These results provide evidence that long-term systemic challenge with Porphyromonas gingivalis, an oral pathogen, can accelerate atherogenic plaque progression. (Circulation. 2002;105:861-867.)

Key Words: atherosclerosis ■ infection ■ cardiovascular diseases ■ Porphyromonas gingivalis

Atherosclerotic coronary artery disease is a contributing factor in 50% of US deaths, half of them caused by thrombosis and myocardial infarction. It is clearly a complex condition with multiple contributing factors. Yet, more than one third of patients dying from atherosclerotic coronary vascular disease do not have any of the classic risk factors.1,2 Recent studies have suggested associations of atherosclerotic risk with infectious agents,1–3 and several pathogens such as Chlamydia pneumoniae, Helicobacter pylori, Cytomegalovirus, herpes simplex virus, Streptococcus sanguis, and Porphyromonas gingivalis have been detected in human atheromas.4–6 A causal relation between these agents and atherosclerosis, however, remains controversial.

Porphyromonas gingivalis (Pg) has been strongly associated with the most common form of periodontal disease: adult periodontitis. Periodontal disease affects an estimated 116 million Americans.7 Recent epidemiological data suggest that periodontal disease is an important risk factor for coronary heart disease, with case-control studies demonstrating a significant association after correction for classic risk factors such as cholesterol, smoking, hypertension, social class, and body mass index.8,9 Proposed explanations include transient bacteremia with spread of infection from the oral cavity; endothelium injury by circulating oral microbial toxins; and systemic inflammation triggered by oral microorganisms.

Pg infection causes local inflammation with gingival ulceration and vascular changes, which increase the incidence and severity of transient bacteremias with gingival trauma.10 Recent in vitro studies have demonstrated that Pg can adhere to and invade endothelial and coronary artery smooth muscle cells.11 This suggests a highly efficient host endocytic uptake of these surface-adherent organisms and the ability of Pg to persist within endothelial cells and potentially to alter their integrity.
It is conceivable that movement of this infection into the systemic circulation with frequent bacteremia could cause a chronic inflammatory insult to the vasculature and contribute to the initiation and progression of atherosclerosis lesions.\textsuperscript{3,12} Although in vitro studies have suggested a possible role for \textit{Pg} in atherosclerosis, it remains to be shown in vivo that long-term \textit{Pg} challenge actually contributes to atheroma lesion development. The purpose of this study was therefore to test the hypothesis that long-term systemic circulatory challenge with \textit{Pg} can promote and accelerate the development of atherosclerotic lesions. To test this hypothesis, we challenged apolipoprotein E-deficient heterozygous (ApoE\textsuperscript{+/−}) mice with \textit{Pg}.

**Methods**

**Bacterial Strain**

\textit{Pg} A7436 was grown on anaerobic blood agar plates (Becton Dickinson Co) in an anaerobic chamber (Coy Laboratory Products Inc) with 85% nitrogen, 5% hydrogen, and 10% carbon dioxide for 3 to 5 days, then inoculated into Schaedler broth (Difco Laboratories) containing hemin and menadione for 24 hours until the culture reached an optical density of 0.8 at 660 nm, corresponding to \(10^{9}\) CFU/mL. Cultured \textit{Pg} and culture medium were diluted with saline in the same dilution before inoculation.

**Mice and Diets**

The Institutional Animal Care and Use Committee of Boston University approved all animal protocols. Male ApoE homozygotes (ApoE\textsuperscript{−/−}, back-crossed for at least 10 generations to the C57BL/6J background) and female wild-type C57BL/6 hybrids were obtained at 8 weeks of age (Jackson Laboratories, Bar Harbor, Me) and fed regular mouse chow. ApoE\textsuperscript{−/−} mice were generated by crossing male ApoE\textsuperscript{−/−} with female wild-type C57BL/6 mice. The experimental protocols are shown in Figure 1. Male ApoE\textsuperscript{−/−} and wild-type mice weaned at 4 weeks of age were randomly assigned to either a high-fat diet (HFD)\textsuperscript{13} (TestDiet) or regular mouse chow (LabDiet). At 10 weeks of age, ApoE\textsuperscript{−/−} or wild-type mice, HFD-fed or chow-fed, were randomly divided into 2 groups: inoculated with live \textit{Pg} (10\textsuperscript{10} CFU/50 \(\mu\)L/mouse) or vehicle containing diluted medium (50 \(\mu\)L/mouse). The choice to use live bacterial inoculation was because in preliminary experiments, we failed to produce atherosclerosis with heat-killed \textit{Pg} (unpublished observation). The inoculation was performed intravenously once per week for 10, 14, or 24 consecutive weeks. The stress caused by injection was minimized and comparable in each group (n=5 per group for each time point).

**Tissue Harvesting and Preparation**

After an overnight fast, mice were heavily sedated with inhaled isoflurane (SOLVAY) and exsanguinated from the femoral arteries. The heart and aorta were perfused for 10 minutes with ice-cold PBS containing 20 \(\mu\)mol/L butylated hydroxytoluene (BHT, Sigma) and 2 mmol/L EDTA, pH 7.4, through a left ventricular cannula. Perfusion was continued for another 10 minutes with cold formal-sucrose solution (10% neutral formalin, 5% sucrose, 20 \(\mu\)mol/L BHT, and 2 mmol/L EDTA, pH 7.4). The aortic tree and proximal aorta were separated close to the heart. The aortic tree, including arch and thoracic and abdominal aorta, was processed for en face analysis\textsuperscript{14} or processed for polymerase chain reaction (PCR) to amplify \(\beta\)-165 ribosomal DNA within samples. The proximal aorta together with the heart was embedded in Histo Prep (Fisher) for cryosections. The liver, spleen, lung, kidney, gall bladder, and small intestine were collected for routine paraffin section and hematoxylin and eosin staining.

**Histomorphometric and Histopathological Analysis of Atheroma Lesions in the Proximal Aorta**

Proximal aortic cross-sections for quantitative and histopathological evaluation of atherosclerotic lesions were prepared as previously described.\textsuperscript{13} Four sections per animal, each separated by 80 \(\mu\)m, were stained (Sudan IV, 35% ethanol and 50% acetone for 6 minutes, and destaining in 80% ethanol for 5 minutes. Total aortic surface area and atherosclerotic lesion area were measured with a stereomicroscope with computer-assisted image analysis capabilities (Image Pro Plus 4.0).

**Immunohistochemical Detection of Macrophages**

Serial cryosections of the proximal aorta were incubated with rat monoclonal antibodies raised against a murine macrophage marker (Mac-3; SD Pharmingen). After blocking endogenous peroxidase and incubation with normal goat serum, sections were incubated with primary antibodies overnight at 4°C. Specimens were then treated with goat anti-rat biotinylated secondary antibodies followed by incubation with streptavidin-peroxidase complex and diamobenzenzene substrate (InnoGenex). Negative controls included incubation with nonspecific rat IgG antibodies in the absence of primary antibodies for each group of sections. Four slides per animal were selected for immunostaining, each separated by 80 \(\mu\)m.

**Serum Levels of Systemic Proinflammatory Mediators**

Systemic inflammatory mediators IL-1\(\beta\) (R&D) and acute phase protein serum amyloid A (SAA) (Tri-Delta Labs) were detected by ELISA assays. Venous blood was collected when the animals were killed. After clotting at room temperature, blood samples were...
centrifuged for 30 minutes at 3500 rpm, and the supernatant was collected and stored at −20°C. Dilutions and determination of standards were done according to the instructions of the manufacturers, and optical density was determined within 30 minutes of colorimetric reaction at 450 nm; corrections were made at 550 nm.

**Pg 16S Ribosomal DNA Amplification by PCR**

DNA was extracted from aorta, heart, and liver tissues (Clontech). The extracted DNA was amplified by a pair of ubiquitous primers corresponding to *Pg*-specific sequences on 16S ribosomal RNA at the base position 729-1132 in a PCR, as described by Slots et al. The 2 primers used to amplify a 404-bp region of 16S ribosomal RNA of *Pg* were 5'-AGG CAG CTT GCC ATA CTG CG-3' and 5'-ACT GTT AGC AAC TAC CGA TGT-3'. PCR was performed for 36 cycles at an annealing temperature of 56°C to 60°C. Amplified products were detected by electrophoresis, and the genomic DNA extracted from *Pg* AT369 served as positive control; PCR performed without template DNA made up the negative control.

**Statistical Analysis**

All of the specimens for en face and histomorphometric measurements were coded so that the measurements were done blindly. All quantitative measurements were confirmed by random re-analysis of approximately one fourth of the specimens by the same examiner (K>0.92) and by another independent examiner (pathologist) to ensure consistency. The intraexaminer and interexaminer variation was <10%. A level of *P*≤0.05 was considered significant. Extent of atherosclerosis was analyzed by ANOVA (2-way) among groups and subsequently by Student’s paired 2-tailed *t* test.

**Results**

**Clinical Assessment**

No clinical signs of infection or mortality were noted in any of the animals at any time. There were no significant differences in body weight between *Pg*-inoculated and noninoculated mice. The heart, kidney, lung, spleen, gall bladder, and small intestine showed normal histological structure, except for mild fatty degeneration in the liver of HFD-fed mice.

**Serum Levels of Systemic Proinflammatory Factors**

Differences among groups at 10 weeks were not significant for IL-1β and SAA. However, significant differences (*P*<0.05) in the levels of both factors appeared at 14 and 24 weeks in *Pg*-injected animals fed chow or HFD. The pattern of SAA expression followed the one observed for IL-1β at all the time points (IL-1β [pg/μL] at 24 weeks: *Pg*-injected 62±31 vs saline-injected 5±2).

**Morphometric Analysis**

**En Face Morphometric Analysis of the Aortic Tree**

In chow-fed groups, the sensitivity of this gross pathological technique did not allow detection of any lesion at any time point. The percent of aortic surface area covered by lesions at all time points was greater in the *Pg*-challenged mice than in vehicle control animals (*P*<0.05) (Figure 2A). In the HFD-fed groups, red-stained lipid-rich lesions were first found 10 weeks after the onset of inoculation with *Pg* but not in vehicle control mice. At 14- and 24-week time points, red-stained lesions were observed in both *Pg*-inoculated and noninoculated mice but to a greater extent in *Pg*-challenged mice (Figure 2B). These lesions were flat or slightly protruding into the vascular lumen and were scattered along the inner curvature of the arch and near the orifices of the intercostal and large abdominal arteries, with the gross appearance of early-stage lesions.

**Histomorphometric Analysis of Atheroma Lesions in the Proximal Aorta**

Lesion cross-sectional area in the proximal aorta increased over time in all animals. In both chow-fed groups (Figure 3A), no lesions were found after 10 weeks of inoculation. Mild foam cell lesions were evident in *Pg*-challenged mice only after 14 weeks of inoculation (*P*<0.01; Figure 3 insets). After 24 weeks of inoculation, the mean lesion area was 9-fold greater in mice challenged with *Pg* than in vehicle control animals (*P*<0.001) and approached a similar level to HFD-fed mice injected with vehicle. In HFD groups, mean lesion area was significantly greater in *Pg*-challenged mice than in unchallenged control mice and became or attained a level 2-fold greater (*P*<0.001) after 24 weeks of inoculation, with the same pattern for percentage of total lumen of the proximal aortas occupied by lesions (Figure 3B).
Histopathological and Immunohistochemical Aspects of Proximal Aorta

Lesions progressed faster in Pg-challenged mice than in vehicle control mice. Also, HFD-fed mice had larger lesions than chow-fed mice. Two stages were observed in the progression of the lesions. The first stage was characterized by sudanophilic lesions somewhat resembling foam cell lesions (Figures 4C, 4D, 5A, and 5B). The lesions related to the aortic valve attachments were more frequent and developed earlier than the lesions in free aortic wall. The second-stage lesions consisted of a mixture of sudanophilic cells, spindle-shaped cells, acellular zones, and an inflammatory infiltrate (Figures 4E and 5, C through F). Stained cells were observed adhering to the surface of the endothelial lining within the lesions. In general, lesions occurred preferentially in 2 locations: the aortic valve attachments (commissures) and the free aortic wall. Staining of macrophages was observed as early as 10 weeks in HFD animals injected either with saline or Pg and intensified with the development of the lesions until 24 weeks. However, staining appeared to be more pronounced in Pg-injected animals than in saline-injected animals. In contrast, chow-fed animals exhibited Mac-3 staining only in Pg-injected groups starting at 14 weeks; immunostaining for Mac-3 followed Sudan IV staining confirmed the presence of macrophages infiltrating between the intima and the media of early lesions.

Atherosclerotic Lesions in Pg-Challenged Wild-Type Mice

Atherosclerotic lesions were not observed in chow-fed wild-type C57BL/6 mice 10 to 24 weeks after Pg inoculation. However, in HFD-fed wild-type mice, only early foam cell lesions were found at 24 weeks after Pg inoculation (Figure 6), comparable to the lesion observed in chow-fed ApoE–/– mice at 14 weeks after Pg inoculation.

Pg 16S Ribosomal DNA Amplification by PCR

No Pg 16S rRNA was amplified from animals at 10 and 14 weeks of Pg injections. However, at 24 weeks, Pg 16S rRNA was amplified in all samples from Pg-injected animals irrespective of their diet.
A, 10 weeks post-inoculation, vehicle injection + HFD

B. 10 weeks post-inoculation, P.g. injection + HFD

C. 14 weeks post-inoculation, vehicle injection + HFD

D. 14 weeks post-inoculation, P.g. injection + HFD

E. 24 weeks post-inoculation, vehicle injection + HFD

F. 24 weeks post-inoculation, P.g. injection + HFD

Figure 5. Cross-sections of aortic lesions in HFD-fed ApoE<sup>-/-</sup> mice challenged with P<sub>g</sub> or vehicle. First-stage lesions (foam cells) adjacent to valve attachments (A and B) and in free aortic wall (B) are found in 10-week postinoculation mice; second-stage lesions are observed in 14- and 24-week postinoculation mice (C through F). Lesions in P<sub>g</sub>-inoculated ApoE<sup>-/-</sup> mice are larger and more advanced than in noninoculated mice at same time point. *Foam cell. L indicates aortic lumen; M, media; and A, adventitia.

Discussion

The association between periodontal disease and atherosclerosis is controversial. Growing evidence from case-control and population-based studies supports an association between periodontal disease and coronary heart disease, but does not define whether the relation is causal or otherwise. In contrast, other studies found no overall<sup>16,17</sup> or only weak associations<sup>18</sup> between periodontal disease and coronary heart disease. The present study was designed to test whether systemic intravascular challenge with an established periodontal pathogen contributes to the development and progression of atherosclerosis in a susceptible animal model. This mode of challenge is reasonable because patients with periodontitis are thought to be chronically exposed to nonsymptomatic bacteremias, the level, duration, and microbial diversity of which increase with periodontal disease severity<sup>3</sup>; dental extraction, periodontal surgery, tooth scaling, and even tooth brushing and flossing can seed oral bacteria into systemic circulation.<sup>10,19</sup> Particularly, bacteremia from a putative pathogen in periodontal disease, P<sub>g</sub>, is observed after dental treatment.<sup>20</sup> For this purpose, we intravenously inoculated live P<sub>g</sub> into heterozygous ApoE<sup>-/-</sup> mice. Unlike ApoE<sup>-/-</sup> mice, in which atherosclerosis spontaneously develops,<sup>21</sup> ApoE<sup>-/-</sup> mice, with only 1 functional ApoE gene, are more susceptible to atherosclerosis than are normal mice (C57BL/6 and 129).<sup>22</sup> In this model, clinical signs of acute systemic illness were not observed in any mice after P<sub>g</sub> inoculation, therefore probably mimicking the chronic nonsymptomatic bacteremias encountered in periodontitis. This model allows us to test the effect of bacterial inoculation while controlling for genetic and dietary factors.

Risk Factor Interaction

The results of this study show that repeated challenge with P<sub>g</sub> inoculation can accelerate the progression of atherosclerosis in an ApoE<sup>-/-</sup> murine model. This acceleration, however, required a genetic susceptibility and/or a dietary risk factor and/or an infectious agent. The importance of genetic susceptibility is reflected in our failure to produce atherosclerotic lesions in chow-fed wild-type C57BL/6 mice after 24 weeks of P<sub>g</sub> inoculation. The importance of diet is highlighted by the earlier, more extensive, and more advanced atherosclerosis lesions observed in ApoE<sup>-/-</sup> mice fed an HFD compared with those fed regular chow. The influence of the infectious agent is illustrated by the fact that wild-type mice fed an HFD had early foam cell lesions after 24 weeks of P<sub>g</sub> inoculation, comparable to those observed in chow-fed ApoE<sup>-/-</sup> mice at 14 weeks of inoculation. In the context of a genetic predisposition such as ApoE<sup>-/-</sup> or an environmental risk factor such as HFD, P<sub>g</sub> infection importantly accelerates the progression of atherosclerosis: We found that aortic atherosclerotic lesions in P<sub>g</sub>-inoculated ApoE<sup>-/-</sup> mice developed earlier and were larger and more advanced than in noninoculated mice with the same genetics and diet. Our data support the contention that the infectious agent alone does not cause atherosclerosis<sup>18</sup> but could contribute to accelerate its progression. Furthermore, long-term P<sub>g</sub> inoculation contributed more significantly than short-term in acceleration of atherosclerosis. At 14-week P<sub>g</sub> inoculation, there were very mild effects in the chow group, whereas at 24 weeks of P<sub>g</sub> inoculation the lesions in chow-fed ApoE<sup>-/-</sup> mice were similar to those of HFD-fed vehicle control group. These data suggest that mild effects of P<sub>g</sub> inoculation could be eventually accumulate, resulting in a remarkable acceleration of atherosclerosis.

Mechanism

The present study demonstrates that P<sub>g</sub>, inoculated weekly, can influence the severity of atherosclerosis. Different pathological mechanisms have been proposed to explain the influence of pathogens in the severity of atherosclerosis.
Direct Vascular Damage

*Chlamydia pneumoniae*,23 Cytomegalovirus,24 and *Herpes virus*25 can accelerate atherosclerosis progression in ApoE−/− mice. *Chlamydia pneumoniae* is a Gram-negative anaerobic microorganism that can invade endothelial and smooth muscle cells,26 can be detected in the human atherosclerotic plaques,4,6 and is currently seen as a risk factor for atherosclerotic disease in case-control studies.3,27 All of these pathogenic characteristics are shared with *Pg* and could constitute the biological mechanisms behind *Pg*-related atherosclerosis. Also, in vitro studies have shown that *Pg* can directly adhere to, invade, and proliferate in aortic and heart endothelial cells and arterial smooth muscle cells,11,28 suggesting that *Pg* may have the arsenal to promote direct aortic cell damage. Since 16S ribosomal RNA was amplified from mouse aortas only at 24 weeks after *Pg* challenge, it is possible that *Pg* has penetrated the lesions only after repetitive inoculations sometime between 14 and 24 weeks. The development of the lesion before this time period could be the result of cumulative effects of systemic inflammation29,30 resulting from upregulation of proinflammatory factors. The amplification of *Pg* 16S rRNA from atherosclerotic lesions does not mean that bacteria are alive and replicating within the lesions, although this possibility should not be ruled out. Furthermore, activated macrophages were recently shown to act as carriers of *Chlamydia* after pulmonary infection, and once established within the lesions, could contribute to local inflammation.31 It is again conceivable that this process could take place in *Pg*-related atherosclerosis; this spreading phenomenon would constitute a passive process, bacteria being only bystanders.32

Microbial Virulence Factors

Recently, Mori and colleagues33 reported that the size of fatty streak lesions was significantly enhanced in mice immunized with human heat-shock protein 60 fed a high-cholesterol diet. Likewise, in normcholesterolemic rabbits, atherosclerosis can be induced by inoculation of bacterial heat-shock proteins.34 Given that *Pg* is particularly rich in heat-shock proteins,35 it is conceivable that they could participate in *Pg*-related atherosclerosis. Our data on heat-killed *Pg* do not support the premise that bacterial virulence factors could contribute to this process but do not completely rule it out, since certain thermosensitive factors could have been destroyed.

Systemic Inflammation

It has been demonstrated that mice lacking the IL-1ra are more prone to atherosclerosis, pointing out a significant role for that cytokine in artery wall damage.36 IL-1β has been also found to be an essential factor in the production of serum amyloid A,37 an acute phase protein released by hepatocytes (the homologue of CRP in mouse) whose concentration rises several hundredfold within 24 hours of inflammatory stimuli.39 Our data show that the IL-1β and SAA were upregulated particularly at 14 and 24 weeks in HFD *Pg*-injected animals. Interestingly, the levels of both markers did not significantly differ at 10 weeks of *Pg* inoculation, suggesting that the prolonged exposure to the bacterial agent produced an amplification of the host response, possibly through activation or enhancement of macrophages.40 It is conceivable that the weekly inflammatory *Pg* stimuli caused the expression of specific systemic proinflammatory factors, raising the overall systemic inflammatory response, therefore predisposing the animals to atherosclerosis. These mechanisms, however, remain unclear and even controversial.

Route of Infection

Although the systemic intravenous route of inoculation does not reflect the natural oral habitat of *Pg*, it does in fact...
reproduce the transient bacteremias that occur in patients with periodontal disease; our hypothesis specifically proposed that such bacteremias act synergistically with classic risk factors to promote atherosclerosis. Nevertheless, the present results pave the way for a better understanding of the biological mechanisms associated with atherosclerosis promoted by infection. Further studies are warranted to mimic Pg oral infection.

In conclusion, our data demonstrate that repetitive systemic challenge with Pg, a known periodontal pathogen, can accelerate atherosclerosis, and it provides a model for exploring the synergistic effect of systemic infectious challenge with genetic and environmental factors in promoting atherosclerotic vascular disease.

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References

Li et al P gingivalis Infection and Atherosclerosis 867

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In the article, “Porphyromonas gingivalis Infection Accelerates the Progression of Atherosclerosis in a Heterozygous Apolipoprotein E–Deficient Murine Model” by Li et al that appeared in the February 19, 2002, of the journal (Circulation. 2002;105:861–867), the Acknowledgments were incorrect. The corrected Acknowledgments appear below.

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