Oral Infection With a Periodontal Pathogen Accelerates Early Atherosclerosis in Apolipoprotein E–Null Mice

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Objective—Because recent epidemiologic evidence suggests that periodontal infections may increase the risk of atherosclerosis and related events in humans, we assessed the impact of oral inoculation with the periodontal pathogen *Porphyromonas gingivalis* on atherogenesis in hypercholesterolemic apolipoprotein E–null mice.

Methods and Results—In the absence of alterations in distinct risk factors, *P gingivalis* infection exacerbated the early stages of atherogenesis in this model. Infected animals displayed evidence of local periodontal infection, as the severity of alveolar bone loss, the hallmark of periodontitis, was increased. Generalized activation of host inflammatory responses was evident in infected mice, as demonstrated by serum IgG response to *P gingivalis* and elevated levels of interleukin-6. *P gingivalis* DNA was localized in the aortic tissue from a limited number of infected mice but not in any noninfected controls. Infected mice displayed enhanced vascular activation, as suggested by increased aortic expression of vascular cell adhesion molecule-1 and tissue factor.

Conclusions—Oral infection with *P gingivalis* accelerates early atherosclerosis. Thus, uncovering the underlying mechanisms is critical for the design of preventive and therapeutic strategies targeting atherosclerotic vascular disease and its sequelae. (Arterioscler Thromb Vasc Biol. 2003;23:1405-1411.)

Key Words: periodontitis ■ atherosclerosis ■ infection ■ *Porphyromonas gingivalis* ■ apoE-null mouse

Periodontal disease is a chronic infection affecting the tissues surrounding and supporting the teeth, caused by the bacteria of dental plaque. It begins as gingivitis, an inflammation of the soft tissues only, and can progress to periodontitis, where destruction of connective tissue attachment and alveolar bone can eventually lead to tooth loss. The global prevalence of periodontal diseases is high, and the severe forms of chronic periodontitis affect approximately 10% to 15% of individuals in most populations.1 Evidence from recent epidemiologic studies suggests a link between periodontal infections and increased risk of atherosclerosis and related cardiovascular and cerebrovascular events in human subjects.2–5 In some studies, associations were observed between the degree of coronary atherosclerosis and alveolar bone loss or the number of missing teeth, suggesting that the degree of vascular activation correlates with periodontal disease severity.3,4 Hypotheses to account for these observations are multiple, including systemic inflammation primed by periodontal infection and release of lipopolysaccharide into the periphery; activation of circulating inflammatory cells and endothelial cells; direct invasion of the vessel wall by oral bacteria consequent to bouts of transient bacteremia; exaggerated, hyperinflammatory responses to periodontal bacteria or cross-reactivity between microbial and human antigens; and underlying predisposing mechanisms, such as genetic predilection.

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These concepts are of particular relevance, because mechanisms involved in the development and progression of atherosclerosis are complex. It is well established that distinct events, such as hyperglycemia, hyperhomocysteinemia, and hypertension, for example, may magnify vascular inflammation and augment processes involved in innate lipid-driven atherogenesis.6–8 In addition to these amplifying factors, multiple studies have suggested a role for infection with a range of organisms, such as *Chlamydia pneumoniae*, cytomegalovirus, and others.9–11 As the precise role of these infections in the development and progression of atherosclerosis is not fully understood, it is logical to propose that chronic periodontal infections may also participate in this multiple hit model, accelerating mechanisms involved in atherogenesis.

Consistent with this premise, a recent report has suggested that large inocula of the major periodontal pathogen *Porphyromonas gingivalis* delivered via weekly intravenous injections into the systemic circulation of heterozygous apolipoprotein E (apoE) +/− mice accelerated atherosclerosis.12
In these experiments, *P. gingivalis* and its key components had highly facilitated access to the systemic circulation. As this mode of delivery of the microorganism did not involve oral inoculation, the means by which *P. gingivalis* ordinarily gains access to the tissues in human subjects, the goal of the present studies was to test whether introduction of *P. gingivalis* into the oral cavity would modulate early atherogenesis in mice that spontaneously develop atherosclerosis. To address these concepts, we subjected apoE-null mice, in which spontaneous hypercholesterolemia leads to formation of atherosclerotic lesions similar in morphology and distribution to those in humans, to topical oral inoculation with *P. gingivalis*. Our murine model of experimental periodontal infection is distinct from models that do not introduce *P. gingivalis* into the oral cavity but rather via parenteral routes. Therefore, our studies directly address the role of oral periodontal infections in modulation of atherogenesis.

**Methods**

**Oral Infection in Apolipoprotein E–Deficient Mice**

All animal studies were performed in accordance with the policies of the Institutional Animal Care and Use Committee at Columbia University. Fifty male homozygous apoE-null mice (backcrossed for more than 10 generations into the C57BL/6 background) were obtained from Jackson Laboratories and housed in a pathogen-free environment. At age 5 weeks, mice received 4 daily ampicillin/kanamycin administrations by gavage (2 mg per drug per day) in an attempt to facilitate the subsequent bacterial inoculation and enhance colonization. Mice were transferred to a non-pathogen–free environment, and 25 of them were inoculated with the human periodontal pathogen *P. gingivalis*, strain 381. Approximately 0.2 mL of 1.5×10^10^ bacterial cells/mL in 2% carboxymethylcellulose with PBS was administered via oral topical application and anal topical application/gavage over a 3-week period for a total of 15 inoculations. Mice are coprophagic, and the rationale behind the latter mode of inoculation is that bacteria will be in the feces and will then return to the mouth, thereby establishing a cycle of oral reinfection. Twenty-five control apoE-null mice received antibiotics and vehicle (sterile PBS) on the same schedule. Body weight was recorded for animals in both groups throughout the experimental period (at 5, 9, 13, and 15 weeks of age). At age 17 weeks, all mice were euthanized.

**Quantification of Atherosclerotic Lesion Area**

Mice were deeply anesthetized, and blood was collected into heparinized syringes from the inferior vena cava. For each mouse, the heart was gently perfused through the pulmonary vein with 0.5 mL of citrate buffer (0.05 mol/L, pH 7.4). On euthanasia, the aorta was retrieved in its entirety to the iliac bifurcation and was snap frozen for analyses. The heart was stored in buffered formalin (10%), and cryostat sections were prepared. Atherosclerotic lesion area was quantified using a Zeiss microscope interfaced with a Sony video camera and an image analysis system (Media Cybernetics), and mean lesion area per mouse was calculated by an investigator blinded to the experimental protocol. Atherosclerotic lesion complexity (fatty streaks versus complex lesions, the latter defined by presence of fibrous caps, cholesterol clefts, or necrosis) was also assessed.

**Quantification of Alveolar Bone Loss**

Evaluation of the severity of periodontal destruction by measurement of alveolar bone loss on the lingual surfaces of posterior mandibles was determined as previously described. Alveolar bone loss was defined as the total area in pixel units between the cementoenamel junction and the alveolar bone crest.

**Lipoprotein and Serum Analysis**

Levels of total cholesterol and triglycerides were determined before euthanization in fasted mice (no caloric intake from 8 to 12 AM) using chromogenic assays (Sigma Chemical Co and Boehringer Mannheim, respectively). Fast Performance Liquid Chromatography (Amersham Pharmacia) was performed using 2 Superose 6 columns run in series onto which pooled sterile filtered mouse plasma was applied (5 mice per condition per assay). Samples from blood collected at euthanasia were also assayed for interleukin (IL)-6 by ELISA (R&D Systems), glucose by a blood glucose meter (Accu-Check Advantage, Roche Diagnostics Corporation), insulin by ELISA (Crystal Chem Inc), and creatinine (Analytix Inc).

Assessment of serum IgG antibody response to *P. gingivalis* was performed by means of the checkerboard immunoassay. Protein-A standards (2.5 μg/mL) and bacterial suspensions of *P. gingivalis* 381 (ATCC 33277) and, as a control, a common oral streptococcal strain not associated with periodontitis (*Streptococcus oralis*, ATCC 35037) were prepared (10^10^ cells/mL) and immobilized on nitrocellulose membranes. Mouse serum samples (diluted 1:300), as well as mouse IgG standards (2000 and 1000 ng/mL) were loaded perpendicularly and were allowed to interact. The membrane was incubated with horseradish peroxidase–conjugated anti-mouse IgG. The ECL detection system (Amersham Pharmacia) was used, and the chemiluminescent signals were assessed in a Lumiliser Workstation (Boehringer Mannheim).

**Immunoblotting**

Aortic tissue was homogenized in lysis buffer (50 mmol/L Tris, pH 7.4, NaCl 0.15 mol/L, phenylmethanesulfonyl fluoride, PMSF, 1 mmol/L), and approximately 20 to 25 μg of total protein per sample was analyzed by SDS-PAGE and immunoblotted with goat anti-human vascular cell adhesion molecule-1 (VCAM-1) IgG (Santa Cruz Biotechnology) and goat anti-rat tissue factor IgG (kindly provided by Dr Vijay Rao, University of Texas Health Sciences at Tyler). Corresponding secondary horseradish peroxidase–conjugated antibodies (Sigma) and the ECL detection system (Amersham Pharmacia) were used for visualization by autoradiography. Densitometry was performed using the ImageQuant software (Molecular Dynamics).

**Immunohistochemistry**

The following primary antibodies were used on paraffin-embedded heart sections (5 μm thick): goat anti-rat tissue factor IgG, rat anti-mouse Mac-3 IgG (Pharmingen/BD Biosciences), and mouse anti-human smooth muscle actin IgG (Dako).

**Polymerase Chain Reaction**

Polymerase chain reaction (PCR) was used on DNA from aortic tissue (DNEasy tissue kit, Qiagen) using published primers and conditions. Standard housekeeping genes for PCR (G3PDH and β-actin) were included to ensure that adequate quantities and quality of isolated DNA were used. Amplified products were detected by electrophoresis. Genomic DNA extracted from *P. gingivalis* 381 served as positive control, and PCR without template DNA served as negative control.

**Statistical Analysis**

Unless otherwise noted, when 2 groups were compared, a Student’s *t* test was used. Statistical analysis was performed using the SAS analysis package (SAS Institute). Parametric correlation analyses were performed, and Pearson’s correlation coefficients were calculated. *P* < 0.05 was considered statistically significant.

**Results**

**Oral Infection Enhances Atherosclerosis and Periodontal Destruction in apoE-Null Mice**

Dissection of the hearts and proximal aortae and macroscopic examination at euthanasia (age 17 weeks) revealed increased
atherosclerotic plaques in \textit{P. gingivalis}–infected versus non-infected apoE-null mice (Figures 1A through 3D). Morphometric analysis revealed that there was a statistically significant 40% increase in mean atherosclerotic lesion area at the aortic sinus in \textit{P. gingivalis}–infected animals compared with PBS-treated controls (Figure 1E; 12,753 \pm 1128 versus 9,080 \pm 951 \text{ mm}^2, n = 25 \text{ group, } P = 0.008).

To determine the degree of local periodontal inflammation/destruction, we dissected the mandibles at euthanasia and measured alveolar bone loss. Infected animals displayed significantly increased alveolar bone loss compared with controls (12,038 \pm 1047 versus 10,978 \pm 1330 pixels; n = 21 mice/group, \( P = 0.007 \)). Suggestive of a potential link between the degree of vascular inflammation and the severity of alveolar bone loss, a trend for a positive correlation (\( r = 0.27, P = 0.08; n = 42 \)) was observed between alveolar bone loss and atherosclerotic lesion area.

Oral Infection and the Systemic Host Response

Activation of host immune responses occurred in infected animals, as demonstrated by increased levels of serum IgG to \textit{P. gingivalis}. Checkerboard immunoblot analysis performed on serum before inoculation revealed no evidence of IgG response to \textit{P. gingivalis} in any of the mice. However, intermediate serum analysis (age 13 weeks, 4 weeks after the last inoculation) revealed a strong IgG response to \textit{P. gingivalis} selectively in infected animals. This response decreased by approximately 10% at euthanasia (age 17 weeks, 8 weeks after the last inoculation). In contrast, at no time point did any of the control animals display an IgG response to \textit{P. gingivalis}, and none of the animals demonstrated a response to \textit{S. oralis}.

In concert with increased serum IgG to \textit{P. gingivalis}, levels of plasma IL-6 by ELISA were significantly increased in infected apoE-null mice versus controls. Horizontal lines represent median values (noninfected, 7.3 pg/mL; infected, 10 pg/mL). Top and bottom of each box mark the first and third quartile of the values (noninfected, 6.2, 10; infected, 7.3, 13.8). Lines extending from the end of each box mark the minimum and maximum for the group (range; noninfected, 5.3 to 22.4 pg/mL; infected, 5.3 to 19.1 pg/mL). Nonparametric analysis, \( P = 0.04, n = 25 \text{ per group.} \)

Pearson’s correlation analysis revealed a statistically significant positive correlation between levels of plasma IL-6 and extent of atherosclerotic lesion area (\( r = 0.37, P = 0.007; n = 50 \)).
and extent of atherosclerotic lesion area was observed (Figure 2B; \( r = 0.37, P = 0.007; n = 50 \)). When only infected mice were included in the correlation analysis (\( n = 25 \)), the correlation coefficient amounted to 0.39 (\( P = 0.05 \)) despite the low number of observations.

**Oral Infection and Bacterial Invasion in the Vessel Wall**

To determine if direct bacterial invasion of the vessel wall may account, at least in part, for the effects of *P. gingivalis* on atherosclerosis, we performed PCR on DNA prepared from aortic tissue collected at euthanasia, 8 weeks after the last inoculation, using published primers specific for *P. gingivalis*. Although none of 9 noninfected mice tested displayed presence of *P. gingivalis* DNA, 2 of 9 infected mice tested demonstrated presence of DNA for this organism. These findings do not unequivocally indicate that bacteria directly invaded the vessel wall but are supportive of this concept.

**Oral Infection and Vascular Activation/Inflammation**

We sought evidence for altered levels of proinflammatory and prothrombotic mediators in the vascular tissue of infected animals. By immunoblotting, levels of VCAM-1 antigen at euthanasia were significantly increased in aortae retrieved from infected mice versus noninfected controls (Figure 3A; \( n = 8 \) per group, \( P = 0.03 \)). Levels of tissue factor antigen in the aorta were increased 4.4-fold in infected versus noninfected mice (Figure 3B, \( n = 4 \) per group; \( P = 0.03 \)). Immunohistochemistry of adjacent sections at the aortic sinus using rat anti-mouse Mac-3 IgG suggested that an important source of tissue factor in the aorta was the macrophage, not smooth muscle cells (the latter as determined using anti-smooth muscle actin IgG) (Figure 4).

**Oral Infection and the Effect on Risk Factors for Atherosclerosis**

Importantly, it was necessary to determine if infection with *P. gingivalis* modulated established risk factors for atherogenesis in this model. No differences in total plasma cholesterol (infected, 444±22 mg/dL; noninfected, 468±15 mg/dL) or triglyceride (infected, 112±6.8; noninfected, 126±7.3) were observed between the 2 groups (Table; \( n = 25 \) per group, \( P = 0.4 \) and 0.2, respectively). However, because it is possible that lack of differences in total lipid levels might belie an altered profile, for instance, a shift toward proatherogenic particles, we separated plasma lipoproteins by size. Fast performance liquid chromatography using sterile-filtered pooled plasma (\( n = 5 \) mice/pool) was performed and revealed no differences between infected versus noninfected mice (Figures 5A and 5B). Body weight was also monitored throughout the experimental period and was similar in infected and noninfected mice at each of 5 assessments.
Importantly, bacteria and release of lipopolysaccharide into the periphery may act as systemic triggers, emerge on infection. Furthermore, such antibodies could, in addition to this premise, it is possible that common underlying predisposing factors, such as genetic predilection of a hyperinflammatory phenotype, emerge as distinct phenotypes in the oral cavity and in the vasculature.

In addition to these indirect means by which periodontitis may prompt vascular perturbation, evidence is emerging that periodontal bacteria, once released into the circulation, may directly invade the blood vessel wall. Indeed, 2 studies in human subjects have suggested that Porphyromonas gingivalis may be localized to atherosclerotic plaques by PCR and immunostaining. Evidence for definitive molecular mechanisms underlying these observations are being uncovered, as studies in vitro suggest that Porphyromonas gingivalis can invade primary cultures of human coronary artery endothelial cells. These observations suggest that once these bacteria adhere to and invade endothelial cells, they may directly activate signal transduction pathways linked to the proinflammatory response.

Our murine model of experimental periodontal infection has advantages over other models that do not introduce Porphyromonas gingivalis into the oral cavity but rather via intravenous or subcutaneous routes. Of course, the limitation of a mouse model is that because of the very small size of the periodontal tissues, direct application of bacteria in the sulci of the animals is impossible. However, there is evidence that periodontal infection occurs in our model, as alveolar bone loss (the hallmark of periodontal disease and the direct result of infection/inflammation locally in the periodontium) was significantly increased in inoculated versus noninoculated mice. In this study, we believe that the microorganism first gained access to the oral epithelium, followed by engagement of systemic host response mechanisms. The only difference between test and control groups of mice is the bacterial inoculation; it is thus reasonable to conclude that the observed effects here are attributable to this bacterial infection. We propose that basal vascular hyperpermeability and dysfunction, primed by elevated levels of circulating lipoproteins, likely facilitated direct invasion of the blood vessel wall in this murine model. In addition, primed vasculature displays enhanced expression of a wide array of adhesion molecules and chemokines, which may additionally trigger and sustain access of Porphyromonas gingivalis to the vessel wall.

Certainly, periodontitis does not ensue from infection by a single microorganism, as the oral environment is very complex with respect to microbial diversity. However, Porphyromonas gingivalis is a most prominent pathogen in chronic periodontitis and has been extensively studied and characterized to date. Therefore, in these first studies, we used oral infection with Porphyromonas gingivalis to address the role of periodontal infections in modulation of atherogenesis. In this context, it is important to note that although the inoculum of bacteria per mouse was high, it is only a small fraction that likely gained access to the

Discussion

In periodontitis, destruction of the attachment apparatus of the teeth is initiated by bacterial colonization of root surfaces and release of bacterial substances onto the epithelial lining of the gingival crevice. Once bacteria gain access to these sites, their byproducts incite periods of exacerbation and remission of inflammation, leading to increased production of proinflammatory cytokines and matrix metalloproteinases, often by augmenting mechanisms linked to evasion of host defenses. A consequence of this heavy burden of cytokines and tissue-destructive mediators is the development of hyperpermeability and loss of epithelial integrity, which creates an opportunity for invading bacteria, and their byproducts, to gain access to the systemic circulation. In addition, it has been demonstrated that events such as toothbrushing, scaling/root planing, extractions, and periodontal surgery may promote haemogenous spread of bacteria.

Definitive support for the concept that oral infection may engage the systemic host response is evident from the observation that specific antibody responses to oral bacteria emerge on infection. Furthermore, such antibodies could, potentially, exert endothelial cytotoxic effects, as reported in the case of antibodies to Escherichia coli and Chlamydia pneumoniae. Importantly, bacteria and release of lipopolysaccharide into the periphery may act as systemic triggers, activating a range of cytokines and tissue-destructive mediators in circulating inflammatory effector cells.

Interestingly, elevated plasma levels of acute-phase reactants such as C-reactive protein, serum amyloid A, fibrinogen, and IL-6 have been reported in periodontitis in human subjects. These observations suggest that recruitment of the systemic inflammatory response machinery may accompany localized diseases of the periodontium. Alternatively, or in addition to this premise, it is possible that common underlying predisposing factors, such as genetic predilection of a hyperinflammatory phenotype, emerge as distinct phenotypes in the oral cavity and in the vasculature.

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epithelia, because the efficiency of local application and gavage is countered naturally by epithelial integrity. Additional studies are needed in this model system to test lower inocula of *P. gingivalis* and different schedules of administration. Such experimentation will be necessary for the study of the precise mechanisms by which this periodontal pathogen seems able to engage the vasculature and, thereby, mediate direct effects on atherogenesis or lesion progression. It is important to emphasize again, however, that evidence linking *P. gingivalis* specifically to vascular/inflammatory cell perturbation is already emerging.\textsuperscript{32–36} Pilot studies from our group have shown that *P. gingivalis* infection of human monocytes incubated with LDL significantly increased foam cell formation compared with LDL incubation alone. DPG3, the fimbriae-deficient mutant of *P. gingivalis* that lacks the ability to adhere to and invade host cells, an important determinant of its pathogenicity in human periodontitis, did not have such an effect.\textsuperscript{37} Furthermore, it is possible that the effects we observed are not specific to *P. gingivalis* and that they might also be induced by other microorganisms. This aspect, as well, is a central focus for future experimentation.

The next step in this research is to use this newly developed animal model to address the mechanistic impact of oral infection at early time points after bacterial inoculation. In this study, our focus was to develop and validate the model; it is highly possible that despite the elevated vascular/plasma indices of inflammation at euthanasia, even additional augmentation of these mediators may have occurred at times more proximal to that of infection.

Lastly, it is well recognized that multiple factors may disrupt vascular homeostasis. In human subjects, elevated levels of lipoproteins and their oxidized forms in particular pose a specific threat. On superimposed perturbation, such as hyperglycemia and its products, increased homocysteine levels, or hypertension, augmentation of vascular dysfunction ensues, leading to acceleration of atherosclerosis and lesion progression, ultimately causing increased incidence of heart attacks and strokes. We propose that the results of the present studies add oral infection with *P. gingivalis* to this array of factors linked to exacerbation of atherosclerosis. Our findings present the first experimental evidence that entry of this periodontal pathogen via the oral route into the host may magnify vascular inflammation and early atherosclerotic lesion formation. These data support the contention put forth by epidemiologic data that unifying mechanisms, at least in part, link the 2 seemingly distinct disorders, periodontal disease and atherosclerosis.

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References


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