Is Porphyromonas gingivalis Cell Invasion Required for Atherogenesis? Pharmacotherapeutic Implications

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Is Porphyromonas gingivalis Cell Invasion Required for Atherogenesis? Pharmacotherapeutic Implications

Salomon Amar, Shou-chieh Wu, and Monika Madan

Various studies have demonstrated an association between chronic bacterial infections and atherosclerotic cardiovascular disease. *Porphyromonas gingivalis*, which can invade endothelial cells, is one pathogen that may link these disorders. If so, antibiotics that block its invasiveness may ameliorate atherosclerotic plaque progression. To explore the role of invasion of *P. gingivalis* in inflammation- and infection-associated atherosclerosis, 10-wk-old ApoE<sup>+</sup> mice were fed either a high fat diet or a regular chow diet. All mice were inoculated i.v., once per week for 24 consecutive wk, with either 50 μl of live *P. gingivalis* (strain 381) (10<sup>7</sup> CFU); a fimbria-deficient *P. gingivalis*; or metronidazole before *P. gingivalis*. Mice were euthanized and evaluated 24 wk after the first inoculation. ApoE<sup>+</sup> mice injected with DPG3 or metronidazole showed significantly fewer atheromatous lesions in the proximal aorta and the aortic tree compared with ApoE<sup>+</sup> than from ApoE<sup>−/−</sup> mice that received either DPG3 or metronidazole before *P. gingivalis* invasion from ApoE<sup>−/−</sup> mice that received *P. gingivalis* alone. Serum cytokine analysis revealed decreased levels of proinflammatory cytokines in both DPG3-injected and metronidazole/P. gingivalis-treated ApoE<sup>−/−</sup> mice compared with mice receiving only *P. gingivalis*, irrespective of diet. *P. gingivalis* invasion is a critical phenomenon in the progression of atherosclerosis. The present data offer new insights into the pathophysiological pathways involved in atherosclerosis and pave the way for new pharmacological interventions aimed at reducing atherosclerosis. The Journal of Immunology, 2009, 182: 1584–1592.

Mounting evidence supports the contention that atherosclerosis is an inflammatory disease. Recent progress in understanding the pathogenesis of atherosclerosis has uncovered central roles for inflammation and infection in the etiology of this most common affliction (1). Furthermore, several reports support a relationship between periodontal infections and certain systemic conditions, including atherosclerosis and cardiovascular disease (2–5). *Porphyromonas gingivalis*, which has been strongly associated with adult periodontitis, is a Gram-negative, nonmotile, obligate anaerobe that can invade and infect epithelium, endothelium, and vascular smooth muscle cells (6–9) and appears to alter endothelial function (10). The capacity for invasion and the subsequent inflammatory responses is in part mediated by fimbriae (11–13), and the virulence of *P. gingivalis* may vary among individual strains depending on which one of the five major types of FimA gene they possess (14).

The epidemiological and initial pathological associations of *P. gingivalis* with atherosclerosis are strengthened in light of the demonstration that *P. gingivalis* can initiate and sustain growth in human vascular cell. TLRs, a group of molecular pattern receptors involved in pathogen recognition, have recently been associated with atherosclerosis (15–17). Elevated TLR expression has been reported at sites of atheroma deposition in both humans and murine models (12, 15). The TLR family of cell surface receptors responds to a variety of microbial structures (18). TLR4 recognizes enteric LPS, whereas TLR2 recognizes peptidoglycan and LPS from organisms that include *P. gingivalis* (19).

Metronidazole (MTZ; 1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole) is an antibacterial and antiprotozoal drug that has been in use for over 35 years. MTZ is active only against anaerobic organisms. It is activated when reduced through electron donation from ferredoxin or flavodoxin that were themselves reduced by the pyruvate:ferredoxin oxidoreductase, possibly forming a hydroxylamine; this process occurs only under strongly reducing conditions (20). Activated MTZ is thought to interact directly with DNA such that the resultant complex can no longer function as an effective primer for DNA and RNA polymerases.

Various pilot clinical trials of preventive antibiotic treatment in patients with coronary disease have shown conflicting results, with some studies supporting (21, 22) and others not supporting (23) the benefit of intervention. To our knowledge, the direct role of MTZ in reducing the progression of atherosclerosis in an in vivo model involving *P. gingivalis* infection has never been studied. We hypothesized that repeat infections with *P. gingivalis* and the addition of a small supplement of dietary cholesterol would yield more consistent and accelerated development of atherosclerotic lesions, and that antibiotic therapy or infection with the fimbria-deficient *P. gingivalis* might inhibit or prevent this process.

Materials and Methods

**Mice and diets**

The Institutional Animal Care and Use Committee of Boston University approved all animal protocols. Apolipoprotein E-deficient homozygote (ApoE<sup>−/−</sup>) mice were obtained from The Jackson Laboratory as breeding and ApoE<sup>+</sup> C57BL/6J males were bred to

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2. Address correspondence and reprint requests to Dr. Salomon Amar, Department of Periodontology and Oral Biology, 650 Albany Street, X-343, Boston University Medical Center, Boston, MA 02118. E-mail address: samar@bu.edu

**Abbreviations used in this paper:** MTZ, metronidazole; HDL, high density lipoprotein; HFD, high fat diet; LDL, low density lipoprotein; SAA, serum amyloid A; VEGF, vascular endothelial growth factor.

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generate ApoE-deficient heterozygotes (ApoE<sup>+/−</sup>), as we previously reported (24). The genotype of all the strains was verified by the PCR method (genotyping protocols from The Jackson Laboratory). ApoE<sup>+/−</sup> mice were weaned at 4 wk of age, and randomly assigned to either cholate-free, a high fat diet (HFD; D12492 from Research Diet), or regular mouse chow (laboratory diet) containing 0.02% cholesterol and 4.5% fat (rodent diet 5001). Because estrogen has been suggested to be cardioprotective in females (25), in this study only male mice were used. Bacterial strain, dose, and route of inoculation

*P. gingivalis* strain 381, a human isolate, and fimbrae-deficient DPG3 were grown on anaerobic agar plates, as described previously (12). Both wild-type *P. gingivalis* and DPG3 were delivered once per week, i.v., at a dose of 10<sup>7</sup> CFU in 50 μl of broth. Control vehicle included the injection i.v. once per week of the same amount (50 μl) of plain uninoculated broth. The dosage and delivery route of the pathogen compare well with bacterial numbers encountered in humans following dental infection, periodontal surgery, scaling, tooth extraction, or flossing (26–28). All animals were 4-wk-old male ApoE<sup>+/−</sup> mice fed either a HFD or a regular chow diet for 6 wk, and then divided into 8 groups of 10 animals that were treated weekly. Group 1 was fed a standard chow diet and inoculated once per week i.v. with 50 μl of broth vehicle; group 2 was fed a standard chow diet and inoculated once per week i.v. with 50 μl of broth vehicle; group 3 was fed a standard chow diet and inoculated weekly with 50 μl of wild-type *P. gingivalis*; group 2 was fed a standard chow diet and inoculated with 50 μl (10<sup>7</sup> CFU) of *P. gingivalis* mutant DPG3; group 3 was fed a standard chow diet and inoculated weekly with 50 μl of broth vehicle; group 4 was fed a HFD and inoculated with 50 μl of wild-type *P. gingivalis*; group 5 was fed a HFD and inoculated with 50 μl (10<sup>7</sup> CFU) of *P. gingivalis* mutant DPG3; and group 6 was fed a HFD and inoculated with 50 μl of broth vehicle. All groups underwent subsequent morphometric and serum analysis after 24 wk of injection in this study.

Animal grouping and experimental time schedule

Experiments involving FimA mutant *P. gingivalis* (DPG3). Animal grouping and experimental time schedule for DPG3 study (Fig. 1) were as follows: at the term of the first 6 wk, animals were divided into 6 groups of 10 animals each. Group 1 was fed a standard chow diet and inoculated with wild-type *P. gingivalis*; group 2 was fed a standard chow diet and inoculated with 50 μl (10<sup>7</sup> CFU) of *P. gingivalis* mutant DPG3; group 3 was fed a standard chow diet and inoculated weekly with 50 μl of broth vehicle; group 4 was fed a HFD and inoculated with 50 μl of wild-type *P. gingivalis*; group 5 was fed a HFD and inoculated with 50 μl (10<sup>7</sup> CFU) of *P. gingivalis* mutant DPG3; and group 6 was fed a HFD and inoculated with 50 μl of broth vehicle. All groups were then divided into two sets for testing two time points, as follows: 14 wk (set 1) and 24 wk (set 2) after the onset of inoculations in this study.

Morphometric analysis

En face morphometric analysis of the aortic tree was as follows: the en face quantification of atherosclerosis in the aortic tree was determined by image analysis, as described previously (4, 30). Data are presented as the percentage of the aorta occupied by the lesions. Briefly, the aortic tree was captured with a Sony color video camera mounted on a stereomicroscope. Sudan red lesions were quantified with a computer-assisted image analysis system (Image Pro Plus 5.0; Media Cybernetics). The extent of atherosclerosis was expressed as a percentage of the aortic surface area covered by lesions (5). Histomorphometric and histopathological analysis of atheroma lesions in the proximal aorta was as follows: proximal aortic cross-sections were prepared for quantitative and histopathological evaluation of atherosclerotic lesions, as described previously (31). Briefly stated, five sections per animal, each separated by 80 μm, were stained (Sudan IV), counterstained (hematoxylin), and assessed with a computer-assisted image analysis system (Image Pro Plus 5.0; Media Cybernetics).
image analysis program (Image ProPlus 5.0). The total cross-sectional area of the aortic lumen and of lesion from the five images was measured. The values were averaged, and then the percentage lesion was expressed as a ratio of total lesion size/total sinu area.

**Lipids and glucose analysis**
Serum samples from all groups of mice (n = 10/group) were evaluated for total cholesterol (fluorometric assay-based kit; Cayman Chemical), high density lipoprotein (HDL), low density lipoprotein (LDL) (colorimetric assay-based kit; Wako Chemicals), and glucose levels (colorimetric assay-based kit; BioAssay Systems). Each sample was analyzed three times in triplicate, according to the kit manufacturer’s instructions.

**ELISA**
Venous blood was collected from mice at the time of euthanasia. After clotting at room temperature, blood samples were centrifuged for 30 min at 2000 × g, and the supernatants were collected and stored at −80°C. Serum amyloid A (SAA) levels were subsequently determined by ELISA (BioSource International), following the manufacturer’s instructions.

**Multianalyte microbead array to detect cytokine expression**
Serum samples from mice euthanized after 24 wk (n = 10/group) were analyzed for inflammatory mediators using a mouse 23-plex and 9-plex cytokine microbead suspension array system (Bio-Plex; Bio-Rad). This microbead array allows for the simultaneous detection of 32 individual inflammatory molecules. These assays were performed three times in triplicate, following the manufacturer’s instructions.

**Statistical analysis**
All histomorphometric measurements were blinded to the examiner. All quantitative measurements were confirmed by random analysis of one-fourth of the specimens by the same examiner (R > 0.92) and by another independent examiner (a pathologist) to ensure consistency. The intraexaminer and interexaminer variation was <10%. All histomorphometric and serum assay data were analyzed by ANOVA, followed by the post hoc Scheffe test. A level of p < 0.05 was considered significant.

**Results**

**Levels of glucose, total serum cholesterol, LDL, and HDL**
Mice were monitored for metabolic status by measuring blood glucose and lipids. The mice injected with broth alone show lower total cholesterol and LDL level, and higher HDL level than the mice injected with wild-type *P. gingivalis*, DPG3 mutant, or wild-type *P. gingivalis* with MTZ (Tables I and II). By comparing the mice injected with wild-type *P. gingivalis* to the mice injected with DPG3 mutant or wild-type *P. gingivalis* with MTZ, we did not observe any significant difference in lipid or glucose profiles between mice injected with wild-type *P. gingivalis* and mice injected with DPG3 within the same diet condition (Table I). Furthermore, total serum cholesterol, LDL, HDL, or glucose levels did not reveal any significant difference between mice treated with MTZ before wild-type *P. gingivalis* and mice injected with *P. gingivalis* alone (Table II).

**En face and histomorphometric analysis of atheroma lesions**
En face analysis did not reveal any lesions in the aortic tree in ApoE/−/− mice injected with wild-type *P. gingivalis* or DPG3 when they had been fed a chow diet. However, among mice fed a HFD, aortic lesions were observed in the mice injected with either wild-type or DPG3 *P. gingivalis*. However, lesion size was significantly less (44.7%; p < 0.05) in the mice injected with DPG3 when compared with wild-type *P. gingivalis* at 24 wk (Figs. 3i and 4a). Histomorphometric analysis of the Sudan-stained lesions revealed a moderate level of lesions in the proximal aorta of chow-fed ApoE/−/− mice injected with wild-type *P. gingivalis*, but no observable lesions in their counterparts injected with DPG3 and broth vehicle (Fig. 3, a–h). Moreover, 53.6% less of the proximal aorta was occupied by the lesions in ApoE/−/− mice fed a HFD and injected with DPG3 when compared with mice fed with HFD and injected with wild-type *P. gingivalis*, and there was no significant difference between ApoE/−/− mice injected with DPG3 and those injected with broth vehicle alone (Fig. 4b).

**The effect of MTZ treatment on progression of atherosclerosis**
In mice fed a HFD, en face analysis demonstrated that administration of MTZ before *P. gingivalis* injection resulted in a 53% reduction in aortic lesions at 14 wk and a 61% reduction (p < 0.05) in aortic lesions at 24 wk, compared with the group receiving *P. gingivalis* injection without MTZ (Figs. 3i and 5a). In mice fed

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### Table I. Metabolic profile

<table>
<thead>
<tr>
<th>Diet</th>
<th>Broth</th>
<th>P. gingivalis</th>
<th>DPG3</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (wk)</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Number of mice (n)</td>
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<td>10</td>
<td>10</td>
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<td>Weight (g)</td>
<td>31.4 ± 3</td>
<td>32.7 ± 6</td>
<td>31.5 ± 9</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>120.0 ± 14</td>
<td>171.7 ± 11</td>
<td>175.8 ± 14</td>
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<tr>
<td>HDL (mg/dl)</td>
<td>78.7 ± 9</td>
<td>41.8 ± 6</td>
<td>43.4 ± 7</td>
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<tr>
<td>LDL (mg/dl)</td>
<td>38.5 ± 5</td>
<td>62.9 ± 13</td>
<td>64.5 ± 10</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>74.2 ± 18</td>
<td>81.3 ± 11</td>
<td>84.7 ± 18</td>
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<tr>
<td>Broth</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
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<td>P. gingivalis</td>
<td>3</td>
<td>10</td>
<td>10</td>
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<tr>
<td>DPG3</td>
<td>46.7 ± 6</td>
<td>47.9 ± 9</td>
<td>48.2 ± 11</td>
<td>&gt;0.05</td>
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<tr>
<td><strong>HFD</strong></td>
<td>1007.1 ± 23.4</td>
<td>1190.3 ± 8</td>
<td>993.3 ± 5</td>
<td>&gt;0.05</td>
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</tbody>
</table>

*Metabolic profile of ApoE−/− mice fed with chow diet or HFD and injected with *P. gingivalis*, DPG3, or broth at 24 wk.

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### Table II. Metabolic profile

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<tr>
<th>Diet</th>
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<th>Metro + P. gingivalis</th>
<th>p Value</th>
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<td>Duration (wk)</td>
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<td></td>
</tr>
<tr>
<td>Number of mice (n)</td>
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<td>10</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
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<td>34.7 ± 6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>171.7 ± 11</td>
<td>164.6 ± 10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>41.8 ± 6</td>
<td>39.7 ± 8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>62.9 ± 13</td>
<td>61.5 ± 11</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>81.3 ± 11</td>
<td>72.3 ± 11</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Broth</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Metro + P. gingivalis</td>
<td>47.9 ± 9</td>
<td>49.9 ± 119</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*Metabolic profile of ApoE−/− mice fed with chow diet or HFD and injected with *P. gingivalis* or MTZ prior to *P. gingivalis* at 24 wk.

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### Table III. Metabolic profile

<table>
<thead>
<tr>
<th>Diet</th>
<th>Broth</th>
<th>P. gingivalis</th>
<th>DPG3</th>
<th>p Value</th>
</tr>
</thead>
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<td>24</td>
<td>24</td>
<td></td>
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<tr>
<td>Number of mice (n)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>32.7 ± 6</td>
<td>34.7 ± 6</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>171.7 ± 11</td>
<td>164.6 ± 10</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>41.8 ± 6</td>
<td>39.7 ± 8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>62.9 ± 13</td>
<td>61.5 ± 11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>81.3 ± 11</td>
<td>72.3 ± 11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Broth</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>47.9 ± 9</td>
<td>49.9 ± 119</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

*Metabolic profile of ApoE−/− mice fed with chow diet or HFD and injected with *P. gingivalis*, DPG3, or broth at 24 wk.
a chow diet, no aortic lesions were observed whether mice were treated with MTZ before P. gingivalis injection or broth alone, whereas mice injected with P. gingivalis alone exhibited significantly higher lesions. Furthermore, no difference in percentage of aortic lesions was observed whether mice received MTZ alone or MTZ before P. gingivalis injection or just broth vehicle alone at either 14- or 24-wk timepoint. However, statistical significance was obtained when animals were injected with P. gingivalis alone (Fig. 5, b and c). Histomorphological analysis of Sudan red-stained lesions revealed a complete lack of aortic lesions in the chow-fed P. gingivalis-injected mice that were treated with MTZ (Figs. 3c and 5b). Administration of MTZ in addition to P. gingivalis to mice in the HFD group resulted in a significant reduction in the percentage of aortic lesions when compared with mice that received P. gingivalis alone: lesions were reduced by 46% (p < 0.05) after 14 wk and by 66% (p < 0.05) after 24 wk (Figs. 3g and 5c). Among mice fed with either chow or HFD, there was no significant difference in lesions in mice receiving MTZ alone when compared with broth alone; thus, mice injected with only MTZ were not included for subsequent analysis (Fig. 5, b and c).

SAA levels

For mice receiving a standard chow diet, there was a significant decrease of 61% (p < 0.05) in SAA levels at 24 wk in ApoE+/– mice injected with strain DPG3 when compared with wild-type P. gingivalis. In mice fed a HFD, there was a 30.1% (p < 0.05) reduction in SAA levels between both groups (Fig. 6a). MTZ treatment of P. gingivalis-injected mice resulted in significant decrease of the serum SAA levels at 24 wk: 80% (p < 0.05) in mice receiving the standard chow diet compared with mice receiving only P. gingivalis, and 49.1% (p < 0.05) in mice fed a HFD (Fig. 6b). Furthermore, the SAA levels of ApoE+/– mice injected with DPG3 were significantly higher (p < 0.05) than those injected with broth vehicle alone, irrespective of diet (Fig. 6a). The SAA

FIGURE 3. a–h. Representative microscopic cross-sections (10 μm) of the proximal aortic root from ApoE+/- mice injected weekly with wild-type, DPG3 mutant P. gingivalis, wild-type P. gingivalis with MTZ, or broth vehicle for 24 wk, and maintained on either a standard chow or a HFD. The sections were stained with Sudan IV and counterstained with hematoxylin to reveal lipid deposition, which was quantified by digital morphometry for ApoE+/- mice. i. Representative aorta from ApoE+/- mice injected weekly with wild-type, P. gingivalis MTZ, broth vehicle, DPG3 mutant P. gingivalis, or wild-type P. gingivalis for 24 wk, and maintained on either a standard chow or a HFD; the progression of the aortic lesions was quantified as percentage of surface area covered.

FIGURE 4. a. Quantitative analysis of the progression of aortic lesions; the data are presented as percentage of aortic surface area covered by lesions in chow-fed ApoE+/- mice and mice fed a HFD and injected weekly with wild-type P. gingivalis or DPG3 for 24 wk. Values represent mean ± SD; *, p < 0.05 for wild-type P. gingivalis in ApoE+/- mice, as compared with DPG3 in ApoE+/- mice. b. Quantitative analysis of lipid deposition in the proximal aortic root; the data presented are the percentage of total lumen of the proximal aorta occupied by lesions in ApoE+/- mice after 24 wk in either the chow-fed group or the HFD group of ApoE+/- mice injected with wild-type, DPG3 mutant P. gingivalis, broth vehicle, or wild-type P. gingivalis with MTZ. Values represent mean ± SD; *, p < 0.05 for wild-type P. gingivalis in ApoE+/- mice, as compared with DPG3 in ApoE+/- mice. Values represent mean ± SD.
level in chow-fed mice treated with wild-type *P. gingivalis* plus MTZ is also significantly higher than the broth-treated group (*p* < 0.05). Interestingly, high fat-fed mice treated with MTZ and injected with *P. gingivalis* showed a 23% decrease (*p* < 0.05) in SAA compared with broth-treated group (Fig. 6b).

**FIGURE 5.** Percentage of aortic surface area covered by lesions in ApoE<sup>−/−</sup> mice fed either chow or a HFD and injected with wild-type *P. gingivalis* with and without MTZ and broth vehicle for 24 wk. *a* Values represent mean + SD; *, *p* < 0.05 for wild-type *P. gingivalis* in ApoE<sup>−/−</sup> mice, as compared with MTZ and *P. gingivalis* in ApoE<sup>−/−</sup> mice. Values represent mean + SD. *b* Microscopic cross-sections (10 μm) of the proximal aortic root were stained with Sudan IV and counterstained with hematoxylin to reveal lipid deposition, which was quantified by digital morphometry for ApoE<sup>−/−</sup> mice. The data represent the percentage of total lumen of the proximal aorta occupied by lesions in chow-fed ApoE<sup>−/−</sup> mice, injected with wild-type *P. gingivalis*, and compared with MTZ treatment and *P. gingivalis* after 14 and 24 wk. Values represent mean + SD; *, *p* < 0.05 for wild-type *P. gingivalis* in ApoE<sup>−/−</sup> mice, as compared with MTZ and *P. gingivalis*. *c* Percentage of total lumen of the proximal aorta occupied by lesions in HFD-fed ApoE<sup>−/−</sup> mice injected with wild-type *P. gingivalis* as compared with mice receiving MTZ and *P. gingivalis* at 14 and 24 wk. Values represent mean + SD; *, *p* < 0.05 for wild-type *P. gingivalis* in ApoE<sup>−/−</sup> mice, as compared with MTZ and *P. gingivalis*.

**FIGURE 6.** SAA levels in serum samples obtained after 24 weekly inoculations, as determined by ELISA in ApoE<sup>−/−</sup> mice maintained on a standard chow or a HFD and injected weekly with wild-type *P. gingivalis*, DPG3, or broth vehicle. *a* Data represent mean + SD; *, *p* < 0.05 for wild-type *P. gingivalis* in ApoE<sup>−/−</sup> mice, as compared with DPG3 in ApoE<sup>−/−</sup> mice. *b* SAA levels in serum samples obtained after 24 weekly inoculations, in ApoE<sup>−/−</sup> mice maintained on either standard chow or a HFD, and injected weekly with wild-type *P. gingivalis*, or MTZ and *P. gingivalis*. Data represent mean + SD; *, *p* < 0.05 for wild-type *P. gingivalis* in ApoE<sup>−/−</sup> mice, as compared with MTZ and *P. gingivalis* in ApoE<sup>−/−</sup> mice. #, *p* < 0.05 for DPG3 or *P. gingivalis* plus MTZ when compared with broth vehicle in ApoE<sup>−/−</sup> mice.

The effect of DPG3 on serum cytokine levels

Mice inoculated with broth alone or DPG3 displayed significantly lower serum cytokine levels for IL-1α, IL-1β, IL-2, IL-6, IL-18, TNF-α, MCP-1, vascular endothelial growth factor (VEGF), and M-CSF than did mice treated with wild-type *P. gingivalis* at 24 wk (Fig. 7), irrespective of
the diet. The mean values from the mice fed a HFD and injected with wild-type *P. gingivalis* were elevated, as follows: TNF-α, $8.87 \times 10^3$ pg/ml; IL-1α, $8.30 \times 10^2$ pg/ml; IL-1β, $2.33 \times 10^3$ pg/ml; and MCP-1, $1.85 \times 10^3$ pg/ml. DPG3-injected mice had lower levels of these cytokines (TNF-α, $4.30 \times 10^3$ pg/ml; IL-1α, $5.61 \times 10^2$ pg/ml; IL-1β, $3.72 \times 10^2$ pg/ml; and MCP-1, $5.78 \times 10^2$ pg/ml). There

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**FIGURE 7.** Serum cytokine levels (pg/ml) in ApoE<sup>−/−</sup> mice fed a standard chow diet and injected weekly with *P. gingivalis*, DPG3, and broth vehicle (*a* and *b*) and mice fed a HFD and injected weekly with *P. gingivalis*, DPG3, and broth vehicle (*c* and *d*). Data represent mean ± SD. *, $p < 0.05$ for wild-type *P. gingivalis* when compared with DPG3 in ApoE<sup>−/−</sup> mice. #, $p < 0.05$ for DPG3 when compared with broth vehicle in ApoE<sup>−/−</sup> mice.

**FIGURE 8.** Serum cytokine levels (pg/ml) in ApoE<sup>−/−</sup> mice fed a standard chow diet and injected weekly with *P. gingivalis*, MTZ plus *P. gingivalis*, or the broth control (*a* and *b*). Cytokine levels are shown from mice fed a HFD and injected weekly with *P. gingivalis*, MTZ, and *P. gingivalis*, or with the broth control (*c* and *d*). Data represent mean ± SD. *, $p < 0.05$ for wild-type *P. gingivalis* when compared with MTZ treatment before *P. gingivalis* in ApoE<sup>−/−</sup> mice. #, $p < 0.05$ for MTZ plus *P. gingivalis* when compared with broth vehicle in ApoE<sup>−/−</sup> mice.
was also a slight increase in the cytokine levels in DPG3-treated mice when compared with the broth-injected mice, irrespective of the diet, with the exception of M-CSF in HFD mice, in which in the broth control group it increased by 11% \((p < 0.05)\) compared with DPG3-injected group. Some cytokines detected in wild-type \(P.\) gingivalis-treated mice were undetectable in DPG3-injected mice. GM-CSF, IFN-\(\gamma\), MIP-1, IL-15, IL-12p40, and IL-12p70 were undetectable in the DPG3-injected group that was fed a chow diet.

The effect of MTZ treatment on serum cytokine levels

MTZ treatment resulted in significantly lower levels of most of the proinflammatory cytokines and chemokines that were up-regulated in response to \(P.\) gingivalis inoculation, irrespective of the diet. Fig. 8, a and b, shows representative proinflammatory cytokines involved in atherosclerosis, including IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-6, IL-18, TNF-\(\alpha\), MCP-1, VEGF, and M-CSF. The cytokine levels in MTZ plus wild-type \(P.\) gingivalis group were found generally higher than broth alone, irrespective of the diet, except in mice fed a chow diet in which IL-1\(\alpha\) and IL-2 demonstrated 34 and 58% increase in broth group compared with MTZ plus wild-type \(P.\) gingivalis group \((p < 0.05)\), and in mice fed a HFD in which IL-2 and M-CSF displayed a 28 and 24% increase in broth group compared with MTZ plus wild-type \(P.\) gingivalis group \((p < 0.05)\), respectively.

Discussion

The mechanism by which infection might induce atherogenesis may be direct or indirect \((32)\). Invasion of the vascular endothelium by pathogenic bacteria such as \(P.\) gingivalis results in the induction of a local inflammatory response, defined by the expression of cell adhesion molecules, TLRs, chemokines, and cytokines \((33)\). These inflammatory molecules have demonstrated significant roles in the initiation and/or acceleration of atherosclerosis.

Recent studies have established that \(P.\) gingivalis invasion of endothelial cells from various tissues \(\)(fetal bovine heart, bovine aorta, and human umbilical vein) is dependent on bacterial fimbriae, as is activation of endothelial responses \((9, 33–35)\). Furthermore, adherence and invasion of the corresponding FimA mutant DPG3, which lacks the major fimbriae, were not detected \((9, 33)\). Khlgatian et al. \((34)\) showed that coculture of HUVEC with \(P.\) gingivalis resulted in the induction in the expression of ICAM-1, VCAM-1, and P- and E-selectins. In contrast, there was no induction of ICAM-1, VCAM-1, or P- or E-selectin expression in HUVEC cultured with the noninvasive \(P.\) gingivalis FimA mutant \((\text{DPG3})\) or when \(P.\) gingivalis was incubated with fimbrillin peptide-specific antisera before the addition to HUVEC. TLRs function as pattern recognition receptors, and have the potential to recognize various virulence factors possessed by \(P.\) gingivalis \((36–38)\). Upon recognition and binding of TLR2 activators, an innate immune response is initiated, leading to activation of transcription factor NF-\(\kappa\)B, which stimulates these cells to produce a variety of innate immune markers, including cell adhesion molecules, TLRs, proinflammatory cytokines, and chemokines. These mediators are believed to be involved in the immunological switch of endothelial cells from a normal antithrombotic to a prothrombotic state. Following invasion/activation of vascular endothelial cells, these cells recruit monocytes, which, in the presence of elevated circulating lipids such as oxidized LDL, form atheromas, a process that may be occurring with \(P.\) gingivalis. Another possible bacterial contribution to vascular disease may be an indirect way in which persistent local infection, such as \(P.\) gingivalis, may cause chronic up-regulation of inflammatory cascades involving TNF-\(\alpha\), IL-1, IFN, IL-8, MCP-1, and C-reactive protein. These cytokines, chemokines, and acute-phase mediators could be released into the vasculature from periodontal focal \(P.\) gingivalis infection. Once in the circulation, these mediators may subsequently activate vascular endothelial cells and contribute to aggravate atherosclerotic lesions.

Two independent approaches were taken to determine the relative importance of systemic inflammation vs direct bacterial invasion of the arterial wall for development of atherosclerotic lesions. The first approach was to use noninvasive \(P.\) gingivalis FimA mutant \((\text{DPG3})\) and directly compare it with wild-type \(P.\) gingivalis. It is noteworthy that by en face and histomorphometric analysis, DPG3 challenge did not induce any lesions in the aorta and the aortic sinus, whereas wild-type \(P.\) gingivalis did. An additional contribution of diet was observed, although mice fed a HFD and injected with DPG3 displayed significantly smaller lesions when compared with mice on the same diet challenged with wild-type \(P.\) gingivalis. We also observed significantly lower SAA serum levels in mice injected with DPG3 when compared with mice injected with wild-type \(P.\) gingivalis, irrespective of the diet, suggesting a lower systemic inflammatory condition. Indeed, cytokine profiling revealed that DPG3 exhibits a significantly reduced ability to induce proinflammatory cytokines when compared with wild-type \(P.\) gingivalis. There was also a significant decrease in the serum cytokine levels of IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-18, TNF-\(\alpha\), MCP-1, VEGF, and M-CSF in DPG3-injected mice when compared with wild-type \(P.\) gingivalis-injected mice. Similar results were observed in mice fed a HFD and injected with DPG3 when compared with mice injected with the wild-type \(P.\) gingivalis strain. DPG3 induced much lower levels of these cytokines as compared with their corresponding controls. The reduced cytokine levels in the DPG3-challenged group support several other in vitro studies that have shown that invasive \(P.\) gingivalis can induce inflammatory cytokines IL-1\(\alpha\), IL-1\(\beta\), IL-6, and TNF-\(\alpha\) in gingival fibroblasts, epithelial cells, and macrophages \((39–41)\). Finally, previous findings indicating that FimA lacking DPG3 is unable to properly produce adhesion molecules involved in cell invasion \((34)\) and only able to up-regulate 4 of 68 up-regulated inflammatory genes by wild-type \(P.\) gingivalis in human aortic endothelial cells also suggest a correlation between FimA-dependent cell invasiveness and the pathogenesis of cardiovascular disease \((13)\). Therefore, in addition to the invasion deficiency, DPG3 lacks FimA, a known potent TLR2 ligand, and pathogenicity may be reduced to only LPS \((36–38, 40)\). Our recent investigation corroborated by other laboratories shows that TLR2\(-/-\) mice exhibit substantially reduced lesions of \(P.\) gingivalis-induced atherosclerosis compared with TLR2\(+/-\) and TLR2\(-/-\) mice, and mice injected with FSL-1, a TLR2 agonist, also showed a similar increase in atherosclerotic lesion size, irrespective of the diet \((4, 42–44)\). The reduction of atherosclerosis observed when animals were infected with DPG3 may not be the result of only lack of invasion capability, but also lack of cell surface-initiated signaling by the FimA.

The second approach was designed to further explore the significance of bacterial invasion in the progression of atherosclerosis, by treating mice with MTZ before \(P.\) gingivalis challenge. The rationale behind using MTZ is that it is the most commonly used antibacterial drug for periodontal and other anaerobic infections \((45, 46)\). MTZ had been previously demonstrated to prevent bacterial invasion of cells and tissues \((47, 48)\). Antibiotic prevention assays are used to characterize the invasion capabilities of bacterial strains in the cells, and are quantified by determining the CFUs recovered following antibiotic treatment. We therefore tested whether MTZ treatment could reduce atherosclerotic plaque development in \(P.\) gingivalis-infected ApoE\(+/-\) mice by preventing
bacterial invasion. Our data show complete prevention of P. gingivalis-associated atherosclerotic lesions both in the aorta and the aortic sinus lesions in mice maintained on a chow diet, and significantly smaller aortic lesions in mice fed a HFD when compared with the wild-type P. gingivalis-injected mice. These findings are best explained by attributing an important role to P. gingivalis invasion in the atherosclerotic process and a preventive or therapeutic role to MTZ. The most probable mechanism of action of MTZ on P. gingivalis is an overall disruption of DNA replication and transcription, leading to cell death. It is noteworthy that MTZ treatment led to reduced SAA levels in the CSF, and VEGF. Compared with DPG3 experiments, MTZ treatment led to reduced levels of cytokines, which are most commonly implicated in atherosclerosis, including IL-1α, IL-1β, TNF-α, IL-6, IL-2, MCP-1, IFN-γ, M-CSF, and VEGF. Compared with DPG3 experiments, MTZ treatment disrupted P. gingivalis invasion, whereas cell surface-initiated signaling by the FimA or LPS via TLR was still active. Yet, MTZ treatment led to slightly more reduced lesions (although not statistically significant) than those observed with DPG3, probably due to the slightly greater suppression systemic inflammatory response by MTZ therapy compared with DPG3, as observed for SAA, IL-1β, and IL-18. Interestingly, our previous data shows that the genetic removal of IL-6, such as in IL-6-deficient mice, was found to aggrivate atherosclerosis when animals were fed a HFD and/or treated with P. gingivalis, therefore concluding that physiological production of IL-6 is necessary for an atheroprotection (4), whereas excessive levels may be detrimental. However, the treatment of mice with DPG3 or MTZ did not induce elevated levels of IL-6 compared with P. gingivalis treatment alone, but instead some IL-6 was still produced in mice treated with DPG3 or MTZ. This IL-6 may be the result of pathogen-associated molecular patterns, such as LPS for DPG3 and LPS and/or FimA for MTZ still active in these two experimental conditions.

The data suggest that invasion and concomitant activation of the endothelium are critical phenomena in initiation and progression of atherosclerosis. Overall, the present data support the hypothesis that fimbrinated P. gingivalis stimulates inflammatory cells to produce mediators (RANTES and MCP1) that may signal the recruitment of inflammatory cells to the site of P. gingivalis infection, as we have shown in previous study (50). Furthermore, once these bacteria adhere to and invade endothelial cells, they may directly activate signal transduction pathways, thereby amplifying the inflammatory response and cell apoptosis. Infection-activated atherosclerosis may be limited to those pathogens that can gain access to the vasculature, interact with vascular endothelium, and activate these cells in a manner that accelerates atheroma formation.

Collectively, this study makes an effort to define the role of specific innate immune signaling molecules in response to invasive bacterial infection, and to correlate these responses to putative mechanisms involved in microbially accelerated atherosclerosis.

Disclosures
The authors have no financial conflict of interest.

References


