Nasal immunization with a 40-kDa Outer Membrane Protein of *Porphyromonas gingivalis* Inhibits Atherosclerotic Plaque Accumulation Caused by Oral *P. gingivalis* Infection

Yukiko Koizumi,¹ Tomoko Kurita-Ochiai,¹ Sumito Oguchi,² and Masafumi Yamamoto¹

Departments of ¹Microbiology and Immunology, and ²Internal Medicine, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to:
Masafumi Yamamoto
E-mail: yamamotomasafumi@nihon-u.ac.jp

Abstract
In this study, we assessed the potential of a nasal vaccine against oral *Porphyromonas gingivalis* infection for the prevention of *P. gingivalis*-accelerated atherosclerosis. Apolipoprotein E-deficient spontaneously hyperlipidemic (Apoε⁻⁺⁻) mice were nasally immunized with the 40-kDa outer membrane protein (40k-OMP) of *P. gingivalis* plus cholera toxin (CT) as adjuvant and then challenged orally with *P. gingivalis*. Atheromatous lesions in the proximal aorta of each animal were analyzed histomorphometrically and the serum concentrations of 40k-OMP-specific antibodies were examined. Oral infection of Apoε⁻⁺⁻ mice with *P. gingivalis* resulted in increased alveolar bone loss. Furthermore, the areas of the aortic sinus that were covered with atherosclerotic plaque were increased in those infected mice when compared with non-infected, control mice. However, nasal immunization of Apoε⁻⁻⁻ mice with 40k-OMP plus CT as adjuvant induced significant levels of 40k-OMP-specific serum IgG and salivary IgA antibody responses. Interestingly, when Apoε⁻⁻⁻ mice were nasally immunized with 40k-OMP plus CT before the infection, alveolar bone loss as along with atherosclerotic plaque accumulation in the aortic sinus were significantly reduced. These results suggest that oral infection with *P. gingivalis* accelerates atherosclerosis in Apoε⁻⁻⁻ mice and that 40k-OMP plus CT may be an effective nasal vaccine for the prevention of atherosclerosis accelerated by *P. gingivalis* infection.

Keywords:
atherosclerosis, nasal immunization, outer membrane protein, *Porphyromonas gingivalis*

Introduction
Atherosclerotic coronary artery disease is a contributing factor of death in Western societies; however, as many as 50% of patients with atherosclerosis lack currently identified risk factors such as hypertension, hypercholesterolemia, diabetes, and smoking, suggesting the presence of other contributory mechanisms (1, 2). Recent investigations have increasingly suggested that inflammatoty and immune mechanisms, activated by infectious agents, might be important in the development of atherosclerosis (3, 4). In this regard, several pathogens such as *Chlamydia pneumoniae*, *Helicobacter pylori*, *Cytomegalovirus*, *Herpes simplex virus*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* have been detected in human atheromas (5–7).

Chronic periodontitis threatens oral health by destroying periodontal tissues and thereby causing tooth loss. Because the process of destruction is irreversible, the damage, once done, is permanent. The global prevalence of periodontal disease is high and severe forms of chronic periodontitis affect approximately 15% of individuals worldwide (8). Furthermore, recent epidemiological studies have suggested that periodontal infection is associated with an increased risk of cardiovascular disease (CVD) (9). For example, patients with periodontitis had a 19% increased risk of CVD compared to subjects without periodontitis (10). It has also been demonstrated that periodontal disease pathogens
reside in the walls of atherosclerosis vessels (11). In addition, DNA from periodontal pathogens including P. gingivalis and Actinobacillus actinomycetemcomitans has been detected in atheromatous plaques (7, 12).

An outer membrane protein having a molecular mass of 40–kDa (40k–OMP) produced by P. gingivalis, one of the major pathogens of chronic periodontitis, is a key virulence factor for coaggregation of P. gingivalis (13–17). Furthermore, this outer membrane protein has been shown to be a hemin-binding protein (18). The 40k–OMP is conserved among many strains of P. gingivalis (15–17). In this regard, previous studies have shown that IgG antibodies induced by the nasal or transcutaneous administration of the 40k–OMP with cholera toxin (CT) as adjuvant inhibited co-aggregation by P. gingivalis (19, 20). Furthermore, transcutaneous immunization with 40k–OMP inhibits abdominal abscess formation by intraperitoneal P. gingivalis challenge (21). These studies suggest that the 40k–OMP might be an effective vaccine for the prevention of P. gingivalis infection.

Apolipoprotein E-deficient spontaneously hyperlipidemic (ApoE<sup>−/−</sup>) mice, an inbred strain created from Japanese wild mice, are deficient in the expression of apolipoprotein E because of a gross disruption of the apoE gene (22, 23). These mice show hypercholesterolemia and accumulate large amounts of remnant-like particles in the bloodstream, as has been observed in apoE<sup>−/−</sup> knockout mice (24). In this study, we used congenic mice with a BALB/c genetic background as an animal model of apolipoprotein E-deficiency to examine the effect of P. gingivalis on the progression of atherosclerosis. Furthermore, the effect of intranasal 40k–OMP on atherosclerosis accelerated by P. gingivalis infection was assessed.

**Materials and Methods**

**Bacterial strain**

P. gingivalis strain 381 was cultured on anaerobic blood agar plates (Becton Dickinson Co., Sunnyvale, CA) with 80% N<sub>2</sub> 10% CO<sub>2</sub> and 10% CO<sub>2</sub> for 3 to 5 days before being inoculated into brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 5 mg/ml hemin and 0.4 mg/ml menadione and grown for 2 days until the culture reached an optical density of 0.8 at 660 nm, corresponding to 10<sup>9</sup> colony-forming units (CFU)/ml. The cultured cells were then centrifuged at 8000 × g for 20 min at 4°C and diluted with phosphate-buffered saline (PBS) for oral infection.

**Mice**

The institutional Animal Care and Use Committee of Nihon University approved all animal protocols. Eight-week-old female BALB/c apolipoprotein E-deficient spontaneously hyperlipidemic (ApoE<sup>−/−</sup>) mice (22, 23), obtained from Japan SLC Inc. (Hamamatsu, Japan), were given regular mouse chow and water ad libitum.

**Antigen and adjuvant**

The recombinant plasmid containing the 40k–OMP gene (pMD125) was kindly provided by Dr. Yoshimitsu Abiko (Nihon University). The 40k–OMP was purified to homogeneity from a cell suspension prepared by sonication of *Escherichia coli* K–12 harboring pMD125, as described previously (25). The purity of the preparation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Furthermore, a *Limulus* amebocyte lysate pyrochrome kit (Associates of Cape Cod, Inc., Woods Hole, MA) was used to determine the level of residual endotoxin. One milligram of the 40k–OMP preparation contained <0.4 pg of endotoxin. Cholera toxin was obtained from List Biologic Laboratories (Campbell, CA).

**Experimental schedule**

The mice were randomly divided into three groups. The first group was non-challenged, whereas the second group was challenged orally five times a week for three weeks with 0.1 ml of live *P. gingivalis* (10<sup>9</sup> cfu/ml). The third group was immunized intranasally three times at weekly intervals with 40k–OMP (10 µg) plus CT (1 µg) prior to the oral challenge with *P. gingivalis*. All mice were monitored daily until they were killed and they appeared
healthy throughout the course of the study. Mice from each group were euthanized at the age of 11 weeks.

*Detection of 40k–OMP–specific serum IgG and saliva IgA antibodies*

Antibody titers in serum and saliva samples were determined by an enzyme–linked immunosorbent assay (ELISA) (26, 27). Briefly, 96–well plates were coated with 40k–OMP (5 μg/ml) and blocked with PBS containing 2% bovine serum albumin. After blocking, serial dilutions of serum or saliva samples were added in duplicate. Starting dilutions of serum and saliva samples were 1 : 2⁰ and 1 : 2², respectively. Following incubation, the plates were washed and peroxidase–labeled goat anti–rat γ or α heavy chain–specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, 2,2’–azino–bis (3–ethylbenz–thiiazoline–6–sulfonic acid) (ABTS) with H₂O₂ (Moss, Inc., Pasadena, CA) was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 414 nm of 0.1 greater than background after 15 minutes of incubation.

*Quantification of the atherosclerotic lesion area*

Blood was collected into heparinized syringes from the orbital veins of mice anesthetized with Isozol (Nichi IKO, Toyama, Japan). The heart and aortic tree were then perfused through the left ventricle with iced 0.9% PBS for 10 minutes. The hearts were then carefully dissected and removed. The upper half of the heart containing the aortic origin was separated and embedded in Tissue–Tek® OCT (optimal cutting temperature) compound (Fisher Scientific, Newark, DE) in cryomolds and cryostat sections were prepared. Using a modified version of the method of Paigen et al (28), we examined cryosections of the aortic sinus for atherosclerotic plaque accumulation by oil red–O staining. The lesion area was then quantified using a microscope interfaced with a CCD (charge–coupled device) camera and an image analysis system (BX51; Olympus, Tokyo, Japan). Briefly, cross–sectional areas from three images were added to obtain the total lesion area per side and the percentage of the aortic lumen that was occupied by lesions per section was calculated. Finally, the total lesion area and the percentage of the aortic lumen occupied by lesions were averaged over four sections per animal and expressed as the mean lesion area and the percentage of the total lumen of the proximal aorta occupied by lesions per section per animal.

*Assessment of alveolar bone loss*

Alveolar bone loss was determined at the maxillary molars of all mice at age of 11 weeks. The right half maxillae were boiled for 1 minute in deionized water, mechanically defleshed, and immersed in 2% (wt/vol) potassium hydroxide (16 h, 25 °C). The half maxillae were then washed and immersed in 3% (wt/vol) hydrogen peroxide (6 h, 25 °C). After the half maxillae were washed, they were stained with 0.1% aqueous methylene blue as described previously (29). Linear measurements were obtained from the cementum–enamel junction to the alveolar bone crest from the maxillary molars at 14 landmark sites per mouse.

*Polymerase chain reaction detection of P. gingivalis*

Whole blood was collected from 11–week–old mice. Total DNA was isolated using a QiaAmp kit (Qiagen, Tokyo, Japan). The extracted DNA was amplified by a pair of ubiquitous primers corresponding to *P. gingivalis*–specific sequences on 16S ribosomal RNA at the base position 729–1132 by the polymerase chain reaction (PCR) as described previously (30). The primers used to amplify a 404–bp region of 16S ribosomal RNA of *P. gingivalis* were 5’–AGGCAGCTTGCCATCTGCG–3’ and 5’–ACT–GTTAGCAACTCCGGT–3’. Amplification by PCR was performed for 40 cycles at an annealing temperature of 57 °C. Amplified products were detected by electrophoresis and the genomic DNA extracted from *P. gingivalis* 381 served as the positive control; amplification performed without template DNA made up the negative control.


Statistical analysis

The data are presented as the mean±standard deviation. One-way ANOVA (analysis of variance) followed by Tukey-Kramer multiple comparison test was used to assess differences in total atherosclerotic plaque accumulation. *p<0.05 was considered significant.

Results

Clinical assessment

No clinical signs of infection or mortality were noted in any of Apoe<sup>−/−</sup> mice at any time. There were no significant differences in body weight between the *P. gingivalis*- and sham-inoculated mice. The heart, kidneys, spleen, liver, and small intestine of each animal showed normal histological structure (data not shown).

Nasal immunization with 40k-OMP induces *P. gingivalis*-specific antibody production

In the initial study, we sought to determine whether nasal administration of 40k-OMP plus CT could induce 40k-OMP-specific antibody responses in Apoe<sup>−/−</sup> mice. The mice nasally immunized with 40k-OMP plus CT showed significant 40k-OMP-specific serum IgG and salivary IgA antibody responses. In contrast, negligible amounts of serum IgG and salivary IgA anti-40k-OMP antibodies were produced by the non-immunized animals (Fig. 1). As expected, administration of CT alone did not induce 40k-OMP-specific antibody responses (data not shown).

Nasal immunization with 40k-OMP reduces enhancement of atherosclerosis caused by *P. gingivalis* infection

In the next study, we determined whether antibodies induced by nasally administered 40k-OMP were capable of reducing the atherosclerosis accelerated by *P. gingivalis* infection. Eleven weeks after the oral infection with *P. gingivalis*, atherosclerotic plaque accumulation was increased when compared with the non-infected mice (Fig. 2). Furthermore, the bacteria was detected in the peripheral blood of those infected mice (Fig. 3). These results indicate that oral infection of *P. gingivalis* resulted in inva-

![Fig. 1. The 40k-OMP-specific antibody responses following nasal vaccination with 40k-OMP plus cholera toxin. Apoe<sup>−/−</sup> mice were nasally immunized with 10 μg of 40k-OMP plus 1 μg of cholera toxin on days 0, 7 and 14 (black bar), or phosphate-buffered saline (open bar). Serum and saliva samples were collected on day 21 and assessed for 40k-OMP-specific serum IgG (A) and saliva IgA (B) antibodies. The results are expressed as the mean±SD for five mice per group. *p<0.01; **p<0.05 compared to the control group. N.D.: not detectable.](image)
Fig. 2. Atherosclerotic plaque formation in the aortic sinuses of Apoe<sup>−/−</sup> mice nasally immunized with 40k-OMP plus cholera toxin prior to an oral challenge with <i>P. gingivalis</i>. The proportion of lesion areas in the aortic sinus is shown (A). The results are expressed as the mean ± SD of five mice per group. *<i>p</i> < 0.01 compared to the control group. **<i>p</i> < 0.05 compared to the group challenged orally with <i>P. gingivalis</i>. Oil red-<i>O</i> staining of the proximal aortas in Apoe<sup>−/−</sup> mice are shown (B). The arrow indicates a typical lipid-rich atherosclerotic area stained with oil red-<i>O</i>.

![Graph showing lesion size comparison between control, non-immunized, and immunized groups.](image)

Polymerase chain reaction analysis supported the histomorphological analysis and showed that none of the immunized mice tested positive for <i>P. gingivalis</i> DNA (Fig. 3). These findings indicate that <i>P. gingivalis</i> was eliminated from the blood by nasal immunization with 40k-OMP.

Fig. 3. Detection of <i>P. gingivalis</i> in the blood of Apoe<sup>−/−</sup> mice. Groups of mice were euthanized after oral infection with <i>P. gingivalis</i>. Total DNA was isolated from the blood of the immunized mice, non-immunized mice, and control, non-infected mice, and then <i>P. gingivalis</i>-specific DNA was detected by PCR using <i>P. gingivalis</i> 16S' specific primers. Lane 1: Purified <i>P. gingivalis</i> 381 gene as a positive control. Lanes 2-6: Purified genes from experimental animal blood.

![PCR gel showing DNA bands for control, non-immunized, and immunized samples.](image)

**Analysis of alveolar bone loss in Apo−<i>−</i>/−<i> mice**

Because nasal immunization with 40k-OMP plus CT elicited serum IgG and salivary IgA antibody responses, we sought to determine whether these antibodies were capable of suppressing bone resorption caused by oral infection with <i>P. gingivalis</i>. Thus, mice given 40k-OMP plus CT were infected orally with <i>P. gingivalis</i>. The immunized mice showed a significant reduction in alveolar bone loss caused by <i>P. gingivalis</i> infection (Fig. 4). These findings indicate that nasal immunization with 40k-OMP plus CT provides protection against oral infection by <i>P. gingivalis</i>.
suggested that chronic periodontitis is associated with an increased risk for CVD (33). Recent studies have demonstrated that *P. gingivalis* adheres to and invades endothelial and coronary artery smooth muscle cells and up-regulates atherosclerotic factors such as adhesion molecules, cytokines, and chemokines (34, 35). Furthermore, systemic challenge with *P. gingivalis* induced inflammatory cytokines and accelerated atherosclerosis development in apolipoprotein E-deficient mice (36). On the basis of the above studies, prevention of *P. gingivalis* infection should be relevant for decreasing the risk of atherosclerosis.

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 (4, 37). 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 the evasion of host defenses (38, 39). 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 this regard, it has been shown that specific antibodies against oral bacteria could exert endothelial cytotoxic effects, as reported in the case of antibodies to *Escherichia coli* and *C. pneumonia* (40–43). Importantly, bacteria and the 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.

Although there are several possible mechanisms for the acceleration of atherosclerosis by *P. gingivalis* (44), the prevention of infection with *P. gingivalis* might be the best way to help prevent atherosclerosis altogether. Our results demonstrate that oral challenge with *P. gingivalis* increases alveolar loss.
and atherosclerosis, which can be prevented by nasal immunization with 40k-OMP plus CT. Because nasal immunization induces both serum IgG and salivary IgA immunological responses affecting systemic and mucosal sites, it is considered to be an effective route not only for oral infectious disease but also for systemic disease caused by an oral pathogen. Furthermore, although the oral cavity is a component of the mucosal immune system, it differs from other mucosal compartments in that its local immune responses are both mucosal and systemic. The salivary glands, also part of the mucosal immune system, produce salivary IgA antibodies. In contrast, the IgG antibody-rich crevicular fluid, which flows continuously from the gingival capillaries, is part of the systemic immune system (45). Because P. gingivalis colonizes both subgingival and supragingival biofilms (46-48), the generation of an IgG response in the crevicular fluid could be a more effective and practical way to reduce P. gingivalis colonization. Thus, effective protection against P. gingivalis infection requires both mucosal and systemic antibody responses.

The immunization protocol used in this study was designed to induce specific antibody responses in both mucosal and systemic compartments. We selected nasal administration as the delivery route to induce a significant 40k-OMP-specific antibody response in the mucosal secretions and serum because of its successful track record (49-51). Furthermore, intranasal immunization is a convenient way that does not require a needle and syringes. This may indeed prove to be an effective means of limiting both the development and progression of atherosclerosis. Taken together, our results indicate that an oral challenge with P. gingivalis induces oral bone loss and moves into the systemic circulation, which associated with pathogen-accelerated atherosclerosis, suggesting that immunization rather than antibiotic therapy may be required to control pathogen–accelerated atherosclerosis.

In summary, this study has provided evidence that nasal administration of 40k-OMP with CT elicits 40k-OMP-specific serum IgG and salivary IgA antibody responses. Moreover, 40k-OMP-specific immune responses induced by 40k-OMP plus CT provide protective immunity against alveolar bone loss and acceleration of atherosclerosis caused by P. gingivalis infection. These findings suggest that nasal administration of 40k-OMP should be considered as a candidate vaccine against both oral and systemic diseases caused by P. gingivalis infection.

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References


33. Wu T, Treviranis M, Genco RJ, Dorn JP, Falkner KL,


