Intranasal Immunization with *Porphyromonas gingivalis* Outer Membrane Protein Inhibits *P. gingivalis*-induced Atherosclerosis in C57BL/6 Mice Fed a Hgh-fat Diet

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**Abstract**
Recent epidemiological studies have shown that an association exists between periodontal disease and cardiovascular disease. We have previously reported that long-term *Porphyromonas gingivalis*-challenge accelerated atherosclerosis in normal C57BL/6 mice fed a high-fat diet (HFD). In this study, we assessed the potential of a nasal vaccine for the prevention of atherosclerosis promoted by *P. gingivalis*. Eight-week-old C57BL/6 mice were nasally immunized with the 40-kDa outer membrane protein of *P. gingivalis* (40k-OMP) alone or together with a cDNA vector plasmid encoding Flt3 ligand (pFL) as adjuvant and then challenged intravenously with *P. gingivalis* strain 33277 three times per week for 10 weeks and sacrificed at 19 weeks of age. Atheromatous lesions in the proximal aorta of each animal were analyzed histomorphometrically, and serum concentrations of 40-kDa OMP-specific antibodies, cytokines and C-reactive protein (CRP) were determined. Nasal immunization with 40k-OMP or 40k-OMP plus pFL significantly induced 40k-OMP-specific serum immunoglobulin G antibody response and reduced atherosclerotic plaque accumulation in the aortic sinus and lowered the serum levels of CRP and cytokines compared to nonimmunized animals. These findings suggest that 40k-OMP may be an effective nasal vaccine for the reduction of atherosclerosis accelerated by *P. gingivalis* in normal C57BL/6 mice fed HFD.

**Keywords:** periodontopathic bacteria, periodontal disease, inflammatory cytokine, low-density lipoprotein cholesterol, C-reactive protein

**Introduction**
Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in the Western world. A sedentary lifestyle and Western dietary habits may contribute to this increased risk of developing CVD (1, 2). For example, consumption of a diet rich in saturated fat is positively associated with elevated plasma lipid levels and a state of subacute chronic inflammation, which are important risk factors promoting the onset and development of CVD (3, 4). More specifically, LDL-cholesterol, the inflammatory molecule C-reactive protein (CRP), and inflammatory cytokines are risk factors implicated in the processes leading to atherosclerosis and the occurrence of cardiovascular events (5, 6).

Recently, periodontitis was also shown to increase the risk of cardiovascular disease (CVD) (7), and accumulating evidence suggests that chronic infection with a periodontal pathogen, such as *Porphyromonas gingivalis*, is associated with increased risk of CVD (8, 9). These studies have highlighted the importance of oral care, and vaccine development may contribute to the improved control of oral infectious diseases followed by atherosclerosis prevention. Numerous studies have linked the association of *Actinobacillus actinomycetemcomitans* and *P. gingivalis* to various forms of periodontal disease; thus, these bacteria are considered to be primary per-
iodontal pathogens. The surface components of these bacteria may provide suitable targets for vaccine antigen (Ag). For example, the 40-kDa outer membrane protein of *P. gingivalis* (40k-OMP) is a key virulence factor for coaggregation (10, 11) and hemagglutination (12), and is conserved among many strains (11).

We previously showed that IgG antibodies (Abs) induced by the nasal or transcutaneous administration of 40k-OMP with an adjuvant inhibited coaggregation by *P. gingivalis* (13, 14). Furthermore, nasal or transcutaneous immunization with 40k-OMP prevented alveolar bone loss or abdominal abscess formation in response to a *P. gingivalis* challenge (15, 16). Therefore, the use of 40k-OMP as a mucosal vaccine Ag may be beneficial in the development of a periodontal vaccine and for the preventive therapy of CVD. Bacterial toxins, such as cholera toxin (CT), are commonly used as mucosal adjuvants in animal models; however, toxicity prevents their use in humans (17). Fli3 ligand (FL), a type 1 transmembrane protein, binds fetal liver kinase 2/fms-like tyrosine kinase 3 (Flk2/Flk3) receptor. FL has multiple roles in early hemopoiesis and B lymphopoiesis (18). Interestingly, daily administration of recombinant FL into mice induces Ag–specific immune responses that are comparable to those supported by adjuvant CT (19). Furthermore, nasal delivery of a DNA plasmid encoding FL (pFL) with protein Ag resulted in the induction of Ab responses in both mucosal and systemic sites (20).

Therefore, in this study, we assessed the potential of a combined intranasal vaccine, the 40k-OMP with pFL, to prevent bacteremia followed by the acceleration of atherosclerosis caused by *P. gingivalis*. The results suggest that nasal 40k-OMP plus pFL is an effective vaccine candidate for the induction of protective immunity against atherosclerosis caused by *P. gingivalis* infection concurrent with hyperlipidemia in normal C57BL/6 mice.

**Materials and Methods**

**Bacterial strain**

*P. gingivalis* strain 33277 was cultured on anaerobic blood agar plates (Becton Dickinson, Sunnyvale, CA) in a model 1024 anaerobic system (Forma Scientific, Marietta, OH) with 10% H2, 80% N2, and 10% CO2 for 3 to 5 days. Cultures were then inoculated into brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5 µg of hemin/ml and 0.4 µg of menadione/ml and grown for 2 days until reaching an optical density of 0.8 at 660 nm, corresponding to 10⁶ CFU/ml. The cultured cells were then centrifuged at 8,000 × g for 20 min at 4 °C and diluted with phosphate-buffered saline (PBS) for intravenous (i.v.) infection.

**Antigen and adjuvant**

Plasmid PMD 125 expressing 40k-OMP was kindly provided by Dr. Yoshimitsu Abiko (Nihon University). The 40k-OMP was purified to homogeneity from a cell suspension of *Escherichia coli* K-12 harboring PMD 125, as described previously (21). The purity of the 40k-OMP was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and no contaminating protein bands were noted. Further, an LAL Pyrochrome kit (Associates of Cape Cod, Inc., Woods Hole, MA) was used to determine the level of residual endotoxin. A 1-mg portion of the 40-kDa OMP preparation contained <0.4 pg of endotoxin. The plasmid pORF9–mFLt3L (pFL) consists of the pORF9–mcs vector (pORF) plus the full–length murine FL cDNA gene (Invivogen, San Diego, CA) (20). The DNA plasmid was purified using the GeneElute Endotoxin-Free plasmid kit (Sigma-Aldrich, St Louis, MO).

**Mice**

The institutional Animal Care and Use Committee of Nihon University approved all animal protocols. Eight-week-old female C57BL/6 mice, obtained from Japan SLC, Inc. (Hamamatsu, Japan), were given HFD containing 16.5% fat, 1.25% cholesterol, and 0.5% sodium cholate (F2HFD1; Oriental Yeast Co, Ltd., Tokyo, Japan) and water ad libitum. The mice were randomly divided into four groups (n=5 for each group; Fig. 1). The first group was challenged i.v. three times a week for 10 weeks with 0.1 ml
Fig. 1. Experimental procedure Nine-week-old female C57BL/6 mice were randomly divided into four groups: group 1 was inoculated with 100 μl of PBS (▼), group 2 was inoculated with 100 μl (10^6 CFU) of P. gingivalis (▼), group 3 was immunized with 40-kDa OMP plus pFL (●) and inoculated with 100 μl (10^6 CFU) of P. gingivalis (▼), group 4 was immunized with 40-kDa OMP only (●) and inoculated with 100 μl (10^6 CFU) of P. gingivalis (▼). The immunized mice were nasally vaccinated with 40-kDa OMP plus pFL or 40-kDa OMP alone once a week for 3 weeks prior to the bacterial challenge. The animals were challenged i.v. with P. gingivalis strain 33277 three times a week for 10 weeks. The animals were sacrificed one week after the final challenge.

of PBS, whereas the second group was challenged i.v. three times a week for 10 weeks with 0.1 ml of live P. gingivalis (10^6 CFU/mouse; Fig. 1). The third group was immunized intranasally once a week for 3 weeks with 40k-OMP plus pFL in sterile, pyrogen–free saline prior to the i.v. challenge, whereas the fourth group was immunized intranasally once a week for 3 weeks with 40k-OMP alone prior to the i.v. challenge (Fig. 1). All mice were monitored daily until sacrifice and appeared healthy throughout the course of the study. One week after the last injection, the mice were killed, and the tissue and blood samples were collected.

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 ice-cold 0.9% PBS for 10 min. The heart was then carefully dissected and removed. The upper half of the heart containing the aortic origin was separat-ed and embedded in Tissue-Tek OCT compound (Fisher Scientific, Newark, DE) in cryomolds, and cryostat sections were prepared (22). Using a modified version of the method of Paigen et al. (22), we examined cryosections of the aortic arch for atherosclerotic plaque accumulation by oil red-O staining. The lesion area was then quantified by using a microscope interfaced with a charge–coupled device camera and an image analysis system (BX51; Olympus, Tokyo, Japan). Briefly, cross-sectional areas from three images were summed to obtain the total lesion area per slide, and the percentage of the aortic lumen occupied by lesions per section was calculated. Slides were analyzed in a blinded manner. Finally, the total lesion area and the percentage of the aortic lumen occupied by lesions were averaged over 15 sections per animal and expressed as the mean lesion area and the percentage of the lumen of the proximal aorta occupied by lesions per section per animal.
**PCR detection of P. gingivalis**

Whole blood was collected from 19-week-old mice. Total DNA was isolated by using a QiaAmp kit (Qiagen, Tokyo, Japan). The *P. gingivalis* 16S gene was then detected by PCR as described previously (23). Genomic DNA extracted from *P. gingivalis* 381 was also subjected to PCR as a positive control.

**Detection of 40k-OMP-specific serum IgG, CRP, monocyte chemoattractant protein1 (MCP–1), interleukin-8 (IL–8) and L-selectin**

The Ab titers in serum samples were determined by using enzyme–linked immunosorbent assay (ELISA). Briefly, plates were coated with 40-k-OMP (5 μg/ml) and blocked with PBS containing 2% bovine serum albumin. After blocking, serial dilutions of the serum samples were added in duplicate at starting dilutions of 1:32 and 1:4, respectively. After incubation, the plates were washed, and peroxidase-labeled goat anti–mouse γ heavy-chain–specific Ab (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, ABTS (2,2’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)) with H₂O₂ (Moss, Inc., Pasadena, MD) was added for color development. The endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density of 0.1 greater than background at 414 nm after 15 min of incubation. Each serum sample from blood collected at euthanasia was also screened by using ELISA (R&D Systems, Inc., Minneapolis, MN) for CRP, MCP–1, IL–8 (MIP–2) and L-selectin.

**Results**

**Nasal immunization with 40k-OMP stimulates P. gingivalis–specific Ab production**

Immunization of mice with 40k-OMP or 40k-OMP plus pFL produced a significant 40k-OMP-specific serum IgG response (Fig. 2). In contrast, negligible amounts of 40k-OMP-specific serum IgG were produced by the nonimmunized animals (Fig. 2) and mice that received the adjuvant alone (data not shown).

**Nasal immunization of mice with 40k-OMP prevents P. gingivalis–enhanced atherosclerosis**

Histomorphological analysis was used to calculate the percentage of the aortic lumen occupied by atheromatous lesions. There was a notable increase in atherosclerotic plaque accumulation in the C57/BL6 mice inoculated with *P. gingivalis* compared those of sham control mice (Fig. 3B, 4008 ± 990 μm² vs. 1487 ± 308 μm², p < 0.01; Fig. 3C 2.89 ± 0.73% vs. 0.84 ± 0.21%, p < 0.01). In contrast, nasal immunization with 40k-OMP or 40k-OMP plus pFL significantly reduced atherosclerotic plaque accumulation in the *P. gingivalis*-infected group (Fig. 3B, 4008 ± 990 μm² vs. 1430 ± 339 μm² for 40k-OMP immunized group, p < 0.01 and 4008 ± 990 μm² vs. 1595 ± 381 μm² for 40k-OMP plus pFL immunized group, p < 0.01; Fig. 3C, 2.89 ± 0.73% vs. 1.12 ± 0.02% for 40k-OMP immunized group, p < 0.01 and 2.89 ± 0.73% vs. 1.00 ± 0.20% for 40k-OMP plus pFL immunized group, p < 0.01). Nasal immunization with pFL alone did not reduce atherosclerotic plaque accumulation in the *P. gingivalis*-infected group (data not shown). Complete attenuation of lesion formation was visually observed in *P. gin-
Fig. 3. Nasal immunization with 40k-Da OMP inhibits atherosclerotic plaque formation in the aortic sinuses of C57/Bl6 mice i.v. challenged with P. gingivalis. (A) Oil red O-stained cryosections of the proximal aorta of the mice. The arrow indicates a typical lipid-rich atherosclerotic area stained with oil red O. Histomorphometric analysis of the mean lesion area (B) and the percentage of the aortic sinus occupied by lesions (C) are shown at 19 weeks. Data are expressed as the means ± SE of five mice per group. **p < 0.01 (compared to the control group). *p < 0.01 *p < 0.05 (compared to the P. gingivalis-challenged group).

givalis-infected animals immunized with 40k-OMP or 40k-OMP plus pFL.

Amplification of the P. gingivalis ribosomal 16 S gene by PCR

Porphyromonas gingivalis was detected in the blood of P. gingivalis-challenged mice by using PCR at 19 weeks; four of five mice were positive. In contrast, none of the sham-inoculated or immunized mice, except for one of 40k-OMP immunized mice, positive for P. gingivalis DNA. These results suggested that P. gingivalis may be eliminated from the blood by nasal immunization with 40k-OMP or 40k-OMP plus pFL (Fig. 4).

Serum CRP and cytokine levels

To further analyze the levels of systemic inflammation in infected animals, we measured serum CRP, MCP-1, IL-8 and L-selectin levels in each group of mice at 19 weeks by ELISA. Mice inoculated with P. gingivalis displayed significantly higher serum CRP and cytokine levels for MCP-1, IL-8, and L-selectin.
than sham-inoculated mice (Fig. 5; CRP, 3.9 ± 0.6 vs. 0.7 ± 0.21 ngmL⁻¹, p < 0.01; MCP-1, 1217.3 ± 234.8 vs. 250.0 ± 67.4 pgmL⁻¹, p < 0.01; IL-8, 126.2 ± 24.9 vs. 45.2 ± 13.1 pgmL⁻¹, p < 0.05; L-selectin, 1286.6 ± 65.5 vs. 53.4 ± 16.5 ngmL⁻¹, p < 0.01). In contrast, the rise in the serum CRP and cytokine levels for MCP-1, IL-8, and L-selectin caused by infection with *P. gingivalis* was markedly reduced in mice immunized with 40k-OMP or 40k-OMP plus pFL (Fig. 5; CRP, 3.9 ± 0.6 vs. 0.1 ± 0.01 ngmL⁻¹ for 40k-OMP immunized group, p < 0.01 and 3.9 ± 0.6 vs. 0.1 ± 0.01 ngmL⁻¹ for 40k-OMP plus pFL immunized group, p < 0.01; MCP-1, 1217.3 ± 234.8 vs. 641.9 ± 330.4 pgmL⁻¹ for 40k-OMP immunized group and 1217.3 ± 234.8 vs. 284.7 ± 83.2 pgmL⁻¹ for 40k-OMP plus pFL immunized group, p < 0.01; IL-8, 126.2 ± 24.9 vs. 55.2 ± 14.1 pgmL⁻¹ for 40k-OMP immunized group, p < 0.05 and 126.2 ± 24.9 vs. 38.2 ± 2.3 pgmL⁻¹ for 40k-OMP plus pFL immunized group, p < 0.01; L-selectin, 1286.6 ± 65.5 vs. 347.7 ± 52.3 pgmL⁻¹ for 40k-OMP immunized group, p < 0.01 and 1286.6 ± 65.5 vs. 304.4 ± 11.1 pgmL⁻¹ for 40k-OMP plus pFL immunized group, p < 0.01).

**Discussion**

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 (24, 25). Furthermore, a systemic or oral challenge with *P. gingivalis* has been shown to accelerate the development of atherosclerosis in apo E-deficient (26, 27) and normal mice fed a HFD (28). Therefore, the prevention of *P. gingivalis* infections should be relevant for decreasing the risk of atherosclerosis. Thus, in this study, we assessed the potential of a combined nasal vaccine, 40k-OMP with FL plasmid, to induce an immune response that would protect the host from *P. gingivalis* challenge.

In assessing the efficacy of nasally administered 40k-OMP, we found that 40k-OMP alone induced significant Ag-specific Ab responses in serum of C57/BL6 mice fed a HFD, while 40k-OMP plus pFL as adjuvant further increased the level of the Ab responses. 40k-OMP-specific IgG Abs induced by nasal vaccine significantly reduced atherosclerotic plaque accumulation in the aortic sinus by the cooperation between *P. gingivalis* infection and hyperlipidemia. Furthermore, in mice given 40k-OMP alone or 40k-OMP plus pFL, increased CRP and cytokine levels by *P. gingivalis* were significantly diminished in mice fed a HFD. These results suggest that nasal immunization with 40k-OMP may be an effective Ag delivery system for the induction of protective immune responses against *P. gingivalis*-accelerated atherosclerosis.
Fig. 5. Nasal immunization with 40kDa OMP suppresses serum CRP, MCP-1, IL-8 and L-selectin levels in *P. gingivalis*-challenged mice. Mice from each group were euthanized at the age of 19 weeks and serum sample of each individual mouse was analyzed by ELISA for hsCRP, MCP-1, IL-8 and L-selectin. The data represent the means±SE of five mice per group. **p<0.01, *p<0.05 (compared to the control group). ***p<
0.01, *p<0.05 (compared to the *P. gingivalis*-challenged group).
It is important to note that nasal immunization with 40k-OMP alone elicited significant serum IgG responses. Normally, protein antigen given via the mucosal route without adjuvant has been generally reported to be only a weak immunogen and so has to been presumed to require a mucosal adjuvant to induce Ag–specific Ab responses (29). Indeed, nasal administration of pFL as adjuvant induced significantly higher levels of protective effect against atherosclerotic plaque accumulation, CRP, and cytokine levels than that of 40k-OMP alone in the P. gingivalis–infected groups. Though these results suggest that pFL is an effective adjuvant for nasal 40k-OMP, it would be more prudent not to use to avoid unnecessary side effects induced by pFL. Even without pFL, specific IgG Ab induced by the nasal 40k-OMP significantly diminished atherosclerotic plaque accumulation, CRP, and cytokine levels accelerated by P. gingivalis. These interesting outcomes could be explained by the immunogenicity of 40k-OMP. Indeed, our previous study indicated that transcutaneous immunization (TCI) with 40k-OMP alone induced significant 40k-OMP–specific serum IgG Ab response. Furthermore, serum IgG Ab induced by 40k-OMP alone greatly diminished coaggregation, hemagglutination and abscess formation by P. gingivalis (14, 15). In another study, sublingual immunization of mice with 40k-OMP plus pFL induced significant salivary IgA, as well as serum IgG, responses, and FL protein levels were significantly higher in saliva of mice given 40k–OMP plus pFL when compared with those from mice given 40k–OMP only (30). Mice nasally immunized with a pneumococcal surface protein A (PspA) plus pFL also showed significantly higher levels of PspA–specific secretory–IgA in nasal washes and a nasal vaccine consisting of PspA plus pFL effectively reduced pre–existing Streptococcus pneumoniae in the nasal cavity (31). Therefore, pFL may be more effective as mucosal adjuvant.

Although the oral cavity is an important and characteristic compartment 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, a part of the mucosal immune system, are known to produce salivary IgA antibodies. However, the IgG antibody–rich crevicular fluid, which continuously flows from the gingival capillaries, is part of the systemic immune system and is biologically active in the oral cavity (32). Indeed, our results suggest that serum IgG induced by nasal immunization with 40k-OMP is delivered from systemic immunity. Furthermore, because P. gingivalis colonizes both subgingival and supragingival biofilms (33, 34, 35), generation of IgG responses in crevicular fluid could be a more effective and practical way to reduce P. gingivalis colonization followed by atherosclerosis which arises according to the inflammation induced by P. gingivalis.

Finally, although several possible mechanism may be involved in the acceleration of atherosclerosis by P. gingivalis (36) as a model organism, the prevention of P. gingivalis infection may be an effective way to reduce the induction of atherosclerosis, in addition to periodontitis. Thus, the prevention of periodontitis might be relevant not only for oral but for systemic health as well. In conclusion, our results demonstrate that atherosclerosis and inflammation are accelerated in C57/BL6 normal mice fed HFD after P. gingivalis infection and that these can be prevented by nasal immunization with 40k-OMP. However, whether this is relevant to humans remains to be established. Furthermore, our results indicate that the early activation of inflammatory mediators in response to an infectious challenge may be associated with pathogen–accelerated atherosclerosis, suggesting that immunization may be appropriate to control pathogen–accelerated atherosclerosis.

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