Nasal Vaccination with GroEL plus CpG ODN Inhibits *P. gingivalis*-induced Inflammation and Alveolar Bone Loss

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Introduction

The oral cavity is structurally considered to be an organ that provide double layers of protection simultaneously through secretory immunoglobulin A (S-IgA) from mucosal immunity and immunoglobulin (Ig) G antibodies (Abs) from gingival crevicular fluid (1–3). Although immunization via injection can induce an effective immune responses in the systemic immune systems, but not an antigen (Ag)-specific mucosal immune responses. Therefore, in order to prevent oral infection, it is ideal to induce mucosal immunity and systemic immunity simultaneously.

Chronic periodontitis reduces oral function by causing periodontal tissue destruction and bone resorption. Recently, chronic periodontitis has been reported to cause systemic diseases such as diabetes, aspiration pneumonia, and atherosclerosis, and their relationship has been investigated (4–9). Therefore, prevention of periodontitis is important for oral health and systemic health derived from the oral cavity. Currently 7 to 10 bacterial candidates have been claimed to be putative periodontal pathogens (10, 11). Among them, *P. gingivalis* is a keystone pathogen that is strongly associated with disease progression of chronic periodontitis. The reason for using GroEL as a vaccine antigen is that it can be found in most putative periodontopathic pathogens, including *P. gingivalis*, and it was reported to cross-react among bacterial antigens. Thus far, a positive relationship has been observed between levels of saliva IgA directed against GroEL and
periodontal disease severity(12), and GroEL vaccines derived from P. gingivalis could reduced periodontopathogenic alveolar bone loss induced by multiple bacteria(13). Therefore, we used GroEL as an antigen for nasal vaccine. Maximizing an Ag-specific immune response using an appropriate mucosal adjuvant is important for mucosal immunity(14, 15). CpG ODN interact with TLR9 expressed by antigen presenting cells to induce Th1 and proinflammatory cytokine responses(16, 17). In addition, CpG ODN has been shown to be a potent adjuvant when given orally(18) or sublingually(19).

Nasopharynx-associated lymphoid tissue (NALT) is one of mucosa-related lymphoid tissues in the oral region. Most of the current nasal immunization studies inject vaccine into each nostril and then deliver it effectively to the NALT, probably by normal inhalation. CpG ODN is an efficacious mucosal adjuvant and is widely used for nasal immunization with protein Ag to induce S-IgA Ab responses and protection. Our previous study, a comparison of mucosal pathways in the immune response using a 40-kDa outer membrane protein (40-kDa OMP) from P. gingivalis, suggests that both nasal and sublingual routes are highly effective in preventing periodontal disease than oral route(20). Sublingual immunization has been recently used for allergic diseases, but its mechanisms are still unclear. Thus, nasal immunization targeting NALT is more effective in inducing immune response against periodontal pathogens in the oral cavity due to the small amount of antigen.

In this study, we determined whether GroEL sublingual delivery could induce antibodies at both mucosal and systemic sites. Furthermore, we evaluated the protective effect against inflammation and bone resorption in P. gingivalis infection.

Materials and Methods

Mice

Female BALB/c mice purchased from Sankyo Lab Services (Tokyo, Japan) were maintained in a pathogen-free experimental facility at Nihon University School of Dentistry at Matsudo. Mice received sterile food and water and were aged 7 weeks when used for experiments. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals, Nihon University School of Dentistry at Matsudo (AP16-MD007).

Antigen and adjuvant

Recombinant plasmid containing P. gingivalis GroEL (pRSET B-HSP60) was kindly provided by Dr. K. Yamaizaki (Niigata University). Recombinant GroEL was purified to homogeneity from a cell suspension prepared by sonication of Escherichia coli BL21 (DE3)/pLysS harboring pRSET B-HSP60, as described previously(21, 22). Purity of the preparation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Furthermore, a LAL Pyrochrome kit (Associates of Cape Cod, Woods Hole, MA) was used to determine the level of residual endotoxin. One milligram of the GroEL preparation contained <0.3 pg of endotoxin(23). Synthetic CpG ODN (5-TCCATGACGTTCCTGACGTT-3') was purchased from Sigma-Aldrich (St. Louis, MO) (19).

Immunization and sample collection

We compared of Ag concentrations before nasal immunization. Nasal immunized with 0.3, 3, and 9 µg of GroEL combined with a mucosal adjuvant 10 µg of CpG ODN. The results determined that the optimal concentration of GroEL was 3 µg and the optimal concentration of mucosal adjuvant CpG ODN was 10 µg from our previous studies(data not shown). For nasal immunization, mice were anesthetized with pentobarbital and immunized with a 10µl aliquot (5 µl per nostril) of phosphate-buffered saline (PBS) containing 3 µg of GroEL plus 10 µg of CpG ODN. The control group was treated with 3 µg of GroEL only. Nasal immunization was performed once a week for 3 weeks, and serum, saliva and nasal wash samples were collected 1 week after the last immunization to examine the GroEL-specific Ab responses(Fig. 1A).

GroEL-specific Ab responses

Ab titers in serum and saliva were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with GroEL (5 µg/ml) and blocked with PBS-containing 1% bovine serum albumin (BSA-PBS). After blocking, serial dilutions of serum or saliva samples were added in duplicate. The starting dilution of serum was 2^3–2^18, while that of saliva was 2^2–2^10. Plates were incubated at 4°C for overnight and washed with PBS-Tween. Horseradish peroxidase (HRP)-conjugated anti-mouse IgM (1:5000), IgG (1:5000) and IgA (1:3000) (Southern Biotechnology Associates, Birmingham, AL, USA) diluted by PBS-Tween were then added.
and incubated for 4 h at room temperature. Finally, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) solution (Moss, Inc., Pasadena, MD) was added for color development. Endpoint titers were expressed as the reciprocal log of the last dilution that gave an optical density at 415 nm of 0.1 greater than non-immunized control samples after 15 min of incubation (19).

Quantitative real-time PCR (qPCR)

Total RNA was purified from gingival tissue using an RNeasy Mini kit and treated with DNase I (Qiagen, Germantown, MD, USA) and reverse-transcribed with Oligo (dT) primers using SuperScript® reverse transcriptase (Invitrogen Corp., Tokyo, Japan) to generate cDNA. Quantitative real-time RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara Bio Inc., Otsu, Japan) in accordance with the manufacturer’s protocol. Briefly, the reactions contained 12.5 µL of 2×SYBR Green PCR Master Mix (Takara Bio Inc.), and each primer at 100 nM, and 30 ng of reverse-transcribed RNA. Specific primers for IL-6, TNF-α, HSP60, and GAPDH were supplied by Takara Shuzo (Kyoto, Japan). PCR was performed using the following protocol: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 30 s. Next, a dissociation curve analysis was performed to confirm specificity. The amplification of each gene was performed in triplicate. Target RNA levels were normalized to that of GAPDH mRNA. The sequences of the primers for PCR were as follows:

- IL-6 forward (5′-CCACTTCACAAGTGAGGCT-3′) and reverse (5′-GCAAGTGCATCATCGTGTTCATAC-3′);
- TNF-α forward (5′-GGAGTAGACAAGGTAC-3′) and reverse (5′-TATGGCCCAGACCCTCACA-3′);
- HSP60 forward (5′-TGCTAAGAATGCATGGTTGC-3′) and reverse (5′-AGCTTCGGCTGAGGTTGAGG-3′);
- GAPDH forward (5′-TGTGTCGCTGATGATGAGA-3′).

Fig. 1. Experimental procedure. (A) Seven-week-old BALB/c mice were nasally immunized with 3 µg of GroEL plus 10 µg of CpG ODN once a week for 3 weeks. Serum, saliva and nasal wash samples were collected 1 week after last administration and were assessed for GroEL-specific Ab titers (group 1). As controls, mice were immunized with only 3 µg of GroEL (group 2). (B) After administration for 3 weeks with PBS, mice were orally treated with CMC (group 3) or P. gingivalis (group 4) 5 times per week for 3 weeks. In the immunized group (group 5), P. gingivalis was orally infected 5 times per week for 3 weeks after nasal immunization with 3 µg of GroEL plus 10 µg of CpG ODN for 3 weeks. Mice were sacrificed 4 weeks after final infection for a measurement of alveolar bone loss.
and reverse (5’-TTGCTGTTGAAGTCGCAGGAG-3’).

**Bacterial strain**

*Porphyromonas gingivalis* strain 381 was cultured anaerobically as described previously (24). The bacteria were harvested from brain heart infusion broth (Difco Laboratories) supplemented with hemin (5µg/mL) and menadione (0.4µg/mL) (23, 25).

**Oral infection**

Nasal immunization was performed once a week for 3 weeks and after final immunization, mice were orally infected with *P. gingivalis* as described previously (26) with minor modifications. Briefly, mice were administered $10^9$ CFU of *P. gingivalis* suspended in 100 µl of PBS with 5% carboxymethyl cellulose (CMC) via oral topical application over 3 weeks for a total 15 inoculations. Control groups included sham-infected mice, which received CMC without *P. gingivalis* (27) (Fig. 1B).

**Measurement of alveolar bone loss**

Mice were analyzed using a micro-computed tomography (micro-CT) (Rigaku, Tokyo, Japan) apparatus operated at 90 kV and 160 µA. The voxel resolution was 20µm. These images were reconstructed and observed using three-dimensional (3D) trabecular bone analysis software TRI/3D-BON (Ratoc System Engineering, Tokyo, Japan). The distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) was measured at 7 points for each molar of maxillae as alveolar bone loss, a clinical parameter in periodontology. The distance of 7 points was summed as alveolar bone loss.

**Statistical analysis**

Results are expressed as mean ± standard error of the mean (SE). Statistical significance was determined by unpaired Student’s t test or by one-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test. *P* values of 0.05 was considered statistically significant.

**Results**

**Induction of GroEL-specific Ab responses by mucosal immunization**

We first examined whether the GroEL plus CpG ODN as a mucosal adjuvant would enhance GroEL-specific Ab responses in both mucosal and systemic lymphoid tissues. Mice nasally immunized with the GroEL plus CpG ODN showed significant GroEL-specific serum IgM, IgG and IgA Ab responses 7 days after the final immunization. In contrast, mice treated with GroEL alone did not induce any detectable titer after nasal treatments (Fig. 2A). Further, nasal immunization of GroEL plus CpG ODN induced GroEL-specific IgA Ab responses in saliva and nasal washes. Again, nasal immunization with GroEL alone failed to elicit an antigen-specific IgA response in saliva and nasal washes (Fig. 2B).

**Nasal immunization with GroEL plus CpG ODN regulate inflammatory cytokines in gingival tissue**

To test the effect of GroEL plus CpG ODN on inflammatory status in gingival tissues of mice orally adminis-
tered P. gingivalis, we examined the IL-6, TNF-α and HSP60 mRNA levels in gingival tissues secretion 1 day after the final challenge with P. gingivalis (Fig. 3). P. gingivalis-challenged mice produced increased levels of IL-6, TNF-α and HSP60 mRNA in the gingival tissues. In contrast, the IL-6, TNF-α and HSP60 mRNA levels following P. gingivalis infection were markedly decreased by the nasal immunization with GroEL plus CpG ODN.

**Nasal immunization with GroEL plus CpG ODN reduces alveolar bone resorption following the oral challenge with P. gingivalis**

The mice given GroEL plus CpG ODN as adjuvant were infected orally with P. gingivalis. Mice immunized with GroEL plus CpG ODN showed a significant reduction in alveolar bone loss caused by P. gingivalis challenge (Fig. 4B, 4C). These findings indicate that simultaneous administration of GroEL and CpG ODN in the nasal cavity effectively prevents bone resorption caused by P. gingivalis infection.

**Discussion**

We have shown that nasal administration with GroEL plus CpG ODN enhanced a GroEL-specific immune responses in serum and saliva. Furthermore, alveolar bone resorption caused by P. gingivalis challenge was significantly reduced in mice administered GroEL plus CpG ODN. The other study using rats demonstrated that P. gingivalis GroEL itself has a powerful virulence factor to cause periodontal tissue destruction. In addition, since P. gingivalis GroEL increased the secretion of IL-6 and IL-8, it may be possible that P. gingivalis has take part in osteoclastogenesis via the activation of receptor activator of nuclear factor κ-B ligand mRNA and the inhibition of alkaline phosphatase mRNA in periodontal ligament cells (28). Furthermore, GroEL has been reported to induce the expression of TNFα and IFNγ in human monocytes (29). Therefore, nasal immunization using P. gingivalis GroEL as an immunogen may suppress the pathogenic activity of GroEL that leads to the destruction of periodontal tissue and bone. We proved the effectiveness of nasal immunization of the GroEL plus CpG ODN against P. gingivalis oral challenge. Increased gingival IL-6, TNF-α and HSP60 mRNA levels after P. gingivalis challenge were significantly reduced in the nasally immunized mice. These results suggest that GroEL plus CpG ODN as an adjuvant regulate these inflammatory cytokines, and thus prevent experimental periodontitis caused by P. gingivalis.

Our separate studies have shown that sublingual immunization with same dose of the GroEL without an adjuvant did not elicits a serum IgG and salivary IgA Ab response in mice (19). GroEL-specific Abs were induced without an adjuvant only when immunized sublingually with a high dose of Ag (30 µg) (23). These were the results of sublingual immunization, but we think the same results can be said for nasal immunization. Our results indicate that by using the appropriate amount of mucosal adjuvant, even a small amount of antigen could induce higher levels of serum GroEL-specific IgG Abs. To induce the maximum levels of GroEL-specific immune responses in both mucosal and systemic immune systems,
it is indispensable to use an appropriate mucosal adjuvant.

Nasal vaccination is widely used to prevent mucosal infection. However, it has been reported that antigens and mucosal adjuvant have risk of migrating to the central nervous system (30). In contrast, another study has reported that the deposition of cholera toxin as a mucosal adjuvant in the olfactory tissues does not result in obvious pathological changes in the brain following nasal immunization (31). Although the exact effects of nasally immunized GroEL plus CpG ODN on the central nervous system are not known, other study has reported that CpG ODN is a safe and useful mucosal adjuvant for nasal administration (32).

In summary, our results showed that the nasal immunization with GroEL plus CpG ODN as adjuvant elicits GroEL-specific serum IgG and IgA, as well as saliva IgA Ab responses. Moreover, GroEL-specific immune responses induced by the GroEL plus CpG ODN promoted the host defence against inflammation and alveolar bone resorption caused by P. gingivalis challenge. These findings suggest that nasal immunization with GroEL plus CpG ODN, which effectively induces antibodies against GroEL, is a better way to control P. gingivalis infection.

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