Nasal or Oral Immunization with GroEL Attenuates *Porphyromonas gingivalis*-Induced Atherosclerosis

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Abstract

Periodontal disease is one of the most prevalent chronic inflammation of oral cavity, and has been reported to be associated with atherosclerosis. In this study, we assessed the potential of orally or nasally administered recombinant HSP60 from *Porphyromonas gingivalis* (rGroEL) for prevention of atherosclerosis accelerated by *P. gingivalis*. Apolipoprotein E deficient spontaneously hyperlipidemic (ApoE^{shl}) mice were orally or nasally immunized with GroEL and then challenged intravenously with *P. gingivalis* 381. Atherosclerotic lesions in the proximal aorta of each animal were analyzed histomorphometrically, and the serum concentrations of rGroEL-specific antibodies and cytokines were determined. Although antibody titer sufficient for taking oral or nasal immunity of GroEL under adjuvant-free condition was not obtained, the antibody titers significantly rose by booster effect of *P. gingivalis* infection. Furthermore, mice given rGroEL orally or nasally followed by *P. gingivalis*-challenge possessed significant reduction of atherosclerotic plaque accumulation in aortic sinus and lowered the serum MCP-1 and ox-LDL levels compared to nonimmunized, *P. gingivalis*-challenged mice. These results suggest that oral or nasal immunization with rGroEL could be an effective vaccine for prevention of atherosclerosis accelerated by *P. gingivalis*.

Introduction

Recent studies have suggested that inflammatory and immune mechanisms activated by infectious agents are important in the development of atherosclerosis (1, 2). Several epidemiological studies have revealed that the host immune reaction against persistent infectious pathogens such as *Chlamydia pneumoniae*, *P. gingivalis*, and cytomegalovirus may promote the development of atherosclerosis (3, 4, 5). In particular, immune activation by the pathogen-derived heat shock protein (HSP) GroEL may result in an autoimmune response followed by atherosclerosis via the structural similarity or "molecular mimicry" of host HSP60 and GroEL. Various studies have shown that HSP60 is selectively located in atherosclerotic lesions rather than non-atherosclerotic areas of the arterial wall (6). Furthermore, a positive correlation exists between

anti-HSP antibody levels and the severity of atherosclerosis. High titers of anti-HSP60 antibodies have been found in patients with carotid atherosclerosis, coronary disease, and stroke (7). HSP (GroEL) of *Porphyromonas gingivalis*, a major periodontal pathogen, was also suggested to be a key molecule linking periodontitis as an infectious disease with atherosclerosis as an autoimmune disease (8, 9, 10). The central role of *P. gingivalis* HSP60 in the immunopathogenic mechanism of both periodontitis and atherosclerosis has been demonstrated in the context of immunodominant T- and/or B-cell epitopes (11). A *P. gingivalis* HSP60 vaccine was reported to successfully reduce the level of alveolar bone loss induced by multiple periodontopathogenic bacteria (12), and an anti-*P. gingivalis* HSP60 serum revealed cross-species recognition to exert an opsonophagocytic function against multiple periodontopathogenic bacteria (13). Mucosal administration of relevant autoantigens is an effective method to attenuate autoimmune diseases by inducing an unresponsive state of tolerance (14, 15).

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Therefore, the present study was performed to examine whether the increasing pathogen burden of *P. gingivalis* into blood vessel affects the GroEL antibody levels in apoE^{shl} mice. Further, we examined the role of mucosal delivery of GroEL in modulating inflammatory events and lesion development in atherosclerosis accelerated by *P. gingivalis*.

Materials and Methods

Bacterial strain

P. gingivalis strain 381 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 660nm, corresponding to 10⁹ CFU/ml. The cultured cells were then centrifuged at 8,000 × *g* for 20min at 4°C and diluted with phosphate-buffered saline (PBS) for intravenous (i. v.) infection.

Purification of recombinant GroEL

The recombinant *P. gingivalis* GroEL plasmid (pRSET B-HSP60) was kindly provided by Dr. K. Yamazaki (Niigata University, Niigata, Japan) (16). Recombinant GroEL protein was purified according to the method of Tabeta et al. (17). Briefly, pRSET B-HSP60 was transformed into *Escherichia coli* BL21 (DE3) pLysS and expressed as a polyhistidine-tagged protein with 0.5mM isopropyl-β-D-thiogalactopyranoside (Gibco/BRL Life Technologies, Rockville, MD) induction for 7h. A cell pellet prepared from 20 L of bacterial culture was resuspended in sonication buffer [20mM sodium phosphate (pH 7.4) and 0.5 M NaCl] and disrupted with a sonicator to release a crude lysate containing the recombinant GroEL protein. The crude cell lysate was applied to a column packed with Talon metal affinity resin according to the manufacturer's instructions. After washing with 20mM sodium phosphate buffer (pH 7.4) containing 60mM imidazole, samples were eluted with 20mM sodium phosphate buffer (pH 7.4) containing 500 mM imidazole. Aliquots (1ml) were collected and analyzed by 10% SDS-PAGE with Coomassie brilliant blue staining.

Mice

The institutional Animal Care and Use Committee of

Fig.1 Experimental procedure. Eight-week-old female ApoE^{shl} mice were randomly divided into four groups: group1 was inoculated with 100 µl of PBS (I), group 2 was inoculated with 100 µl (10⁸ CFU) of *P. gingivalis* (I), group 3 was nasally (five times a week) immunized with GroEL (◊), group4 was orally (twice a week for 2 weeks) immunized with GroEL (◊). The animals were challenged i.v. with *P. gingivalis* strain 381 three times a week for 3 weeks. The animals were sacrificed 1 week after the final challenge.

Nihon University approved all animal protocols. Eight-week-old female BALB/c, apoE-deficient spontaneously hyperlipidemic (c. KOR-ApoE^{shl}) mice (18), obtained from Japan SLC, Inc. (Hamamatsu, Japan), were given regular mouse chew and water ad libitum. The mice were randomly divided into four groups (n=6 for each group; Fig.1). The first group was challenged i.v. three times a week for 3 weeks with 0.1 ml of PBS, whereas the second group was challenged i.v. three times a week for 3 weeks with 0.1 ml of live *P. gingivalis* (10⁸ CFU/mouse; Fig.1). The third group was immunized intranasally (19) five times a week with GroEL (30 µg) in sterile, pyrogen-free saline prior to the i.v. challenge, whereas the fourth group was immunized orally (20) twice a week for 2 weeks with GroEL (30 µg) in sterile, pyrogen-free saline 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 10min. The heart was then carefully dissected and removed. The upper half of the heart containing the aortic origin was separated and embedded in Tissue-Tek OCT compound (Fisher Scientific, Newark, DE) in cryomolds, and cryostat sections (6 μ m) were prepared using a modified version of the method of Paigen et al. (21), 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.

Serum analysis

Serum was isolated from the blood by centrifugation at $2500 \times g$ for 20 min after clotting at room temperature. The serum levels of anti-GroEL and anti-HSP60 antibodies were measured by ELISA (22, 23). Briefly, GroEL (5mg/ml) or HSP60 (5mg/ml) (Stressgen Biotechnologies Corporation, Victoria, Canada) was coated onto 96 -well plates. Diluted serum samples were added, followed by peroxidase-conjugated sheep anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Finally, 2,2'-azino-bis (3-ethyl-banz-thiazoline-6-sulfonic acid) with H₂O₂ (Moss Inc., Pasadena, MD) was added for color development. Fifteen minutes after incubation, the endpoint titers were expressed as the reciprocal log₂ of the last dilution that yielded an optical density of 0.1 greater than background at 415nm. Serum samples were also screened by high-sensitivity ELISA for monocyte chemoattractant protein-1 (MCP-1) (R&D Systems Inc., Minneapolis, MN) and Oxidized low-density lipoprotein (Ox-LDL) (CUSABIO Biotech CO., Ltd., Newark, DE, USA).

Fig.2 *P. gingivalis* GroEL-specific and HSP60-specific IgG responses in serum. Apoe^{shl} mice were orally or nasally immunized with GroEL, and then i.v. challenged with *P. gingivalis* as described in the legend for Fig.1. Serum samples were collected at 16 weeks and assessed for GroEL-specific serum IgG antibodies. The results are expressed as the mean $\pm$ SD of six mice per group. *, $P < 0.05$ (compared to the control group).

Statistical analysis

The data are presented as the means $\pm$ the standard errors (SE). Extent of atherosclerosis was analyzed by ANOVA (two-way) among groups and subsequently by Student's paired two-tailed t-test. In all analysis, $p < 0.05$ was taken to indicate statistical significance.

Results

Serum GroEL-specific and HSP60-specific antibodies

Nasal or oral immunization of mice with GroEL produced insufficient GroEL-specific serum IgG response under adjuvant-free condition (Fig.2). On the other hand, anti-GroEL antibody titer rose remarkably by *P. gingivalis*-challenge after immunity. The difference in final antibody titer was not seen at both immunization route. In contrast, GroEL-specific serum IgG antibodies were not detected by the non-immunized mice or *P. gingivalis*-challenged mice (Fig.2). Furthermore, we also analyzed the mean levels of anti-HSP60 antibodies in serum of mice which challenged with *P. gingivalis* and/or immunized with GroEL. Anti-HSP60 antibodies were not detected in any of the group (data not shown).

Histomorphometric analysis of the aortic sinus

A cryosection of the aortic sinus was examined for atherosclerotic plaque accumulation by Oil Red-O staining. Histomorphological analysis was used to calculate the percentage of the aortic lumen occupied by atheromatous lesions. At 16 weeks, there was a notable increase in

Fig.3 Atherosclerotic plaque formation in the aortic sinuses of Apoe^{shl} mice i.v. challenged with *P. gingivalis* or immunized with GroEL prior to the challenge. (A) Oil red-O stained cryosections of the proximal aortas of 16-week-old mice. The arrows indicates typical lipid-rich atherosclerosis areas stained with oil-red O. Histomorphometric analysis of the mean lesion area (B) and the percentage of the aortic sinus occupied by lesion (C) at 16 weeks. The data represent the mean $\pm$ the SD of six mice per group. *, $P < 0.05$ (compared to the control group). #, $P < 0.05$ (compared to the *P. gingivalis*-challenged group).

atherosclerotic plaque accumulations in the Apoe^{shl} mice inoculated with *P. gingivalis* compared to the sham control animals (Fig.3B, $13,525 \pm 2,019 \mu\text{m}^2$ vs. $4,706 \pm 1,091 \mu\text{m}^2$, $p < 0.05$; Fig.3C, $6.62 \pm 0.74\%$ vs. $2.39 \pm 0.73\%$, $p < 0.05$). In contrast, nasal or oral immunization with GroEL reduced atherosclerotic plaque accumulation in the *P. gingivalis*-infected group (Fig.3B, $13,525 \pm 2,019 \mu\text{m}^2$ vs. $9,721 \pm 1,310 \mu\text{m}^2$ for nasal immunized group, $p < 0.05$ and $13,525 \pm 2,019 \mu\text{m}^2$ vs. $8,494 \pm 720 \mu\text{m}^2$ for oral immunized group, $p < 0.05$; Fig.3C, $6.62 \pm 0.74\%$ vs. $4.72 \pm 1.01\%$ for nasal immunized group, $p < 0.05$ and $6.62 \pm 0.74\%$ vs. $4.12 \pm 0.35\%$ for oral immunized group, $p < 0.05$). Nasal or oral immunization with GroEL alone did not increased atherosclerotic plaque accumulation (data not shown). Significant attenuation of lesion formation was visually observed in *P. gingivalis*-infected mice nasally or orally immunized with GroEL (Fig.3A).

Serum MCP-1 and ox-LDL levels

To further analyze the levels of systemic inflammation in the immunized animals, we measured the serum MCP-1 and ox-LDL levels in each group of mice at 16 weeks by ELISA. Serum samples obtained from *P. gingivalis*-challenged mice expressed increased levels of MCP-1 and ox-LDL compared to sham-treated mice (Fig.4; MCP-1, 597.3 ± 58.7 vs. $363.0 \pm 56.8 \text{ pg/ml}$, $p < 0.05$; ox-LDL, 7.1 ± 1.4 vs. $5.1 \pm 1.1 \text{ nmol/ml}$, $p < 0.05$). In contrast, nasal or oral immunization with rGroEL significantly decreased MCP-1 and ox-LDL levels raised by *P. gingivalis* infection. (Fig.4; MCP-1, 597.3 ± 58.7 vs. $317.4 \pm 144.4 \text{ pg/ml}$ for nasal immunized group, $p < 0.05$ and 597.3 ± 58.7 vs. $341.3 \pm 81.7 \text{ pg/ml}$ for oral immunized group, $p < 0.05$; ox-LDL, 7.1 ± 1.4 vs. $4.5 \pm 0.9 \text{ nmol/ml}$ for nasal immunized group, $p < 0.05$ and 7.1 ± 1.4 vs. $4.2 \pm 0.8 \text{ nmol/ml}$ for oral immunized group, $p < 0.05$).

Discussion

The present study showed that increasing the pathogen burden of *P. gingivalis* into blood vessel enhanced inflammation and atherosclerosis in Apoe^{shl} mice without it influences anti-GroEL antibody titer and anti-HSP60 antibody titer, which can be prevented by nasal or oral immunization with GroEL in the system different from immune tolerance.

Previous studies indicate that priming with infectious agents containing homologous HSP60, such as *P. gingivalis*, is involved in plaque formation and progression because of

Fig.4 Serum MCP-1 and ox-LDL levels in *P. gingivalis*-challenged or immunized serum samples collected after 16 weeks. The data represent the mean $\pm$ the SD for six mice per group. *, $P < 0.05$ (compared to the control group). #, $P < 0.05$ (compared to the *P. gingivalis*-challenged group).

the development of autoimmune reactions in the arterial wall (24, 25). Human and microbial HSP namely share a high degree of sequence homology, which consequently may lead to misdirected autoimmunity against human self-HSP expression on stressed cells of blood vessels. On the other hand, several studies have provoked an increased interest in mucosal tolerization against HSP60 aiming to suppress atherogenesis (20, 26). Both of these studies could successfully demonstrate a reduction in aortic plaques by this approach. Therefore, we assessed the implications of the antibodies to GroEL in *P. gingivalis*-induced atherosclerosis, and the potential of mucosal tolerance induction to GroEL.

It has been shown that the increased production of anti-GroEL antibodies with increasing *P. gingivalis* burden was associated with atherosclerosis severity (27). These results, along with the expression of hHSP60 by cells of the

lesion, support the hypothesis of molecular mimicry as a mechanism involved. However, in our study, increasing *P. gingivalis* burden into blood vessel did not enhance the production of anti-GroEL antibodies in serum. The elevated serum antibody titers to GroEL were not detected even in oral infection with *P. gingivalis* (data not shown). Furthermore, even if there was no increase in anti-GroEL antibody titer, the significant increase in inflammation or a plaque formation occurred by *P. gingivalis* infection. Hoymans et al. (28) recently suggested that the antibody responses to Chlamydia HSP60 was significantly lower in patients with coronary atherosclerosis, arguing against the suggestion that infection contributes to disease progression via anti-HSP antibodies. Alfakry et al. (29) also suggested that the carriage of periodontal pathogen in saliva or the periodontal status is not sufficient enough to awaken a systemic HSP60 antibody response considered proatherogenic.

All humans develop protective, beneficial adaptive immunity against the phylogenetically highly conserved microbial HSP60 antigen via infection or vaccination in addition to the immunity against organism-specific epitopes.

In this study, a sufficient antibody titer over an experimental period was not obtained in mice nasally or orally immunized with GroEL only, although it has been reported that GroEL has adjuvant activity (30). Normally, protein antigen given via the mucosal route without adjuvant has been generally reported to be only a weak immunogen and so has to be presumed to require a mucosal adjuvant to induce Ag-specific Ab responses (31). Therefore, it is thought that high antibody titer against GroEL was obtained by the booster effect by *P. gingivalis*-challenge. Further, even without adjuvant, specific IgG Ab induced by the nasal or oral immunization with GroEL followed by *P. gingivalis*-challenge significantly diminished atherosclerotic plaque accumulation, MCP-1, and ox-LDL levels accelerated by *P. gingivalis*.

It has been shown that patients with periodontitis had higher concentrations of circulating HSP60 than controls (32). Further, it appears that there is a very strong correlation between HSP60 and small, dense LDL from the following reasons. To the first, the lectin-like oxidized low-density lipoprotein receptor 1 of macrophage was suggested to be a specific receptor for HSP60 that binds the chaperonin via its C terminus (33). The second, immunization with a combination of HSP60 and apolipoprotein-B

peptide antigen significantly reduced early atherosclerotic lesions in a mouse model (34). The third, HSP60 was found to regulate some functions of oxidized LDL such as modulation of F-actin capping proteins involved in actin polymerization and macrophage motility (35). Since our previous studies also indicate that periodontal pathogens can accelerate atherosclerosis in Apoe^{shl} mice by inducing HSP60 expression in aorta (36, 37), it may say that circulating HSP 60 could play a role in chronic inflammation and could become target for therapeutic measures aiming at reducing the risk of atherogenic dyslipidemia and its complications.

In conclusion, our result showed that the pathogen burden of *P. gingivalis* into blood vessel accelerated atherosclerosis without it increases GroEL antibody titer. This result indicated that the increase of GroEL antibody titer may not be related to aggravation of a disease. Furthermore, since the mucosal immunization with GroEL controlled acceleration of the disease by *P. gingivalis* infection with the increase in GroEL antibody titer, it was suggested that expansion of the inflammation by HSP60 is participating in progress of arteriosclerosis deeply. Furthermore, it was suggested that the mucosal vaccine with GroEL could control the inflammation by periodontopathic bacteria infection and progress of atherosclerosis.

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