Serum Antibodies against *Porphyromonas gingivalis* GroEL are Insufficient to Induce *P. gingivalis*-accelerated Atherosclerosis

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**Abstract**

Periodontitis is associated with an increased risk of atherosclerosis, and accumulating evidence suggests a positive association between anti–heat shock protein 60 autoantibodies and atherosclerosis in humans. Heat shock proteins of the GroEL or HSP60 class are highly conserved proteins essential to all living organisms. In this study, we examined the effects of immunization with recombinant HSP60 from *Porphyromonas gingivalis* on antibody responses and the development of atherosclerosis. Atherosclerosis was examined in BALB/c mice fed a high-fat diet or a regular chow diet following subcutaneous immunization with GroEL or intravenous injection with *P. gingivalis*. The proximal aorta lesion area, serum levels of anti–GroEL antibodies, CRP and MCP-1 levels, expression of HSPs, and inflammatory mediator expression in aorta were measured.

Early atherosclerotic lesion area was substantially lower in HFD-fed mice immunized with GroEL compared to *P. gingivalis*-challenged mice, although significant atherosclerotic lesions, serum immunoglobulin G responses to GroEL, and HSP60 were detected in GroEL-immunized mice. Immunohistochemical staining confirmed strong HSP60 expression in the vascular wall of HFD-fed mice compared to RD-fed mice. Although immunization of the HFD-fed mice with GroEL slightly enhanced serum MCP-1 secretion as well as CD40/40L and LOX-1 expression in the aorta, it did not affect serum CRP levels. These results suggest that immune response cross-reactivity to bacterial HSPs, including periodontal pathogens, with arterial endothelial cells expressing HSP60 are not associated with atherosclerosis severity caused by *P. gingivalis* challenge. This may explain why antibody responses to bacterial HSPs are an unlikely major risk factor for coronary artery disease.

**Introduction**

Although atherosclerotic cardiovascular disease (CVD) is the leading cause of death in Western societies, as many as 50% of patients with atherosclerosis lack currently identified risk factors, including hypertension, hypercholesterolemia, diabetes, and smoking, suggesting the existence of other contributory mechanisms (1, 2). Recently, periodontitis was shown to increase the risk of CVD (3), and accumulating evidence suggests that chronic infection with a periodontal pathogen such as *Porphyromonas gingivalis* is associated with an increased risk of CVD (4, 5). Indeed, recent studies have shown that human atheromatous plaques may contain invasive periodontal pathogens, as well as traces of their DNA (6, 7), further suggesting the role of bacterial infection in the pathogenesis of atherosclerosis. *P.
gingivalis promotes platelet aggregation, increases systemic inflammatory markers, invades endothelial and vascular smooth muscle cells, and appears to alter endothelial function (8). Furthermore, P. gingivalis accelerates the progression of atherosclerosis in homozygous and heterozygous apolipoprotein E (apoE)–deficient mice, rabbits, and pigs (8). We also previously showed that a P. gingivalis challenge led to significant increases in the size and lipid content of atherosclerotic lesions in C57BL/6 mice fed a high-fat diet (HFD) (9).

Recent studies have suggested that inflammatory and immune mechanisms activated by infectious agents are important in the development of atherosclerosis (10, 11). 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 (12–15). 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 (h) HSP60 and GroEL. Various studies have shown that HSP60 is selectively located in atherosclerotic lesions rather than non–atherosclerotic areas of the arterial wall (16). Additionally, 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 (17).

Therefore, the present study was performed to examine whether humoral immune responses induced by a subcutaneous challenge with P. gingivalis GroEL would promote and/or accelerate the development of atherosclerotic lesions comparably to P. gingivalis challenge.

Materials and Methods

Purification of recombinant GroEL

The recombinant P. gingivalis GroEL plasmid (pRSET B–HSP60) was kindly provided by Dr. K. Yamazaki (Niigata University, Niigata, Japan) (18). Recombinant GroEL protein was purified according to the method of Tabeta et al. (19). Briefly, pRSET B–HSP60 was transformed into Escherichia coli BL21 (DE3) pLysS and expressed as a polyhistidine–tagged protein with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Gibco/BRL Life Technologies, Rockville, MD) induction for 7 h. A cell pellet prepared from 20 L of bacterial culture was resuspended in sonication buffer [20 mM 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 20 mM sodium phosphate buffer (pH 7.4) containing 60 mM imidazole, samples were eluted with 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM imidazole. Aliquots (1 mL) were collected and analyzed by 10% SDS–PAGE with Coomassie brilliant blue staining.

Animal experiments

The Animal Care and Use Committee of Nihon University approved all animal protocols. Eight–week–old female BALB/c mice, obtained from Sankyo Lab Services (Tokyo, Japan), were maintained in the experimental facility under pathogen–free conditions. Mice were randomly assigned to receive either a HFD containing 16.5% fat, 1.25% cholesterol, and 0.5% sodium cholate (F2HFD1; Oriental Yeast Co., Ltd., Tokyo, Japan) or a regular chow diet (RD) containing 5.7% fat. The mice were subcutaneously immunized with GroEL (2 or 10 μg per mouse) or phosphate–buffered saline (PBS) in incomplete Freund’s adjuvant at 9, 12, and 15 weeks. Blood samples were collected from the orbital venous plexus under anesthesia at 24 weeks. At 24 weeks of age, the mice from each group (n = 6) were killed, and tissues (from heart to aorta) were excised from each mouse. As a positive control, we used HFD–fed BALB/c mice that were inoculated intravenously (i.v.) with live P. gingivalis (10^8 CFU/100 μL per mouse) three times a week for 10 weeks and sacrificed at 19 weeks of age. In sham control, PBS (100 mL per mouse) was inoculated instead of P. gingivalis.

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 separated and embedded in Tissue–Tek OCT Compound (Fisher Scientific, Newark, DE) in cryomolds, and cryostat sections were prepared (20). Using a modified version of the method of Paigen et al. (20), we examined
cryosections of the aortic arch for atherosclerotic plaque accumulation by Oil Red O staining. The lesion area was then quantified under a microscope interfaced with a CCD camera and an image analysis system (BX51; Olympus, Tokyo, Japan). Briefly, the 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. All 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 × g for 20 min after clotting at room temperature. The serum levels of anti-GroEL and anti-HSP60 antibodies were measured by ELISA (21) (22). Briefly, GroEL (5 mg/mL) or HSP60 (5 mg/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). For competitive adsorption, the serum samples were also incubated for 1 h at 25 °C with 10 mg/mL HSP60 (determined from a pilot study) to absorb any HSP60 antibody activity before being added to the plate. Finally, 2, 2’-azino-bis (3-ethyl-benz-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 415 nm. Serum samples from blood collected at 24 weeks were also screened by high-sensitivity ELISA for C-reactive protein (hsCRP; Kamiya Biomedical Co., Thousand Oaks, CA) and monocyte chemo attractant protein-1 (MCP-1) (R&D Systems Inc., Minneapolis, MN).

Immunohistochemical assessment
Paraffin-embedded sections of the aortic sinus (4 μm thick) were incubated with HSP60 rabbit polyclonal antibodies (1724-1; Epitomics, Burlingame, CA), followed by 3, 3’-dianibobenzidine tetrahydrochloride according to the manufacturers’ instructions. Finally, all sections were counterstained with Mayer’s hematoxylin.

Statistical analysis
The data are presented as the mean ± standard deviation (SD). The extent of atherosclerosis was analyzed by two-way ANOVA among groups and subsequently by Student’s paired two-tailed t-test. In all analyses, P < 0.05 was taken to indicate statistical significance.

Results
Histomorphometric analysis of the aortic sinus
We examined cryosections of the aortic sinus for atherosclerotic plaque accumulation by Oil Red O staining. The average total lesion size and percentage of the lumen occupied by atheroma were determined. Histomorphological analyses revealed that immunization with 2 or 10 μg of GroEL weakly increased the mean atherosclerotic lesion area at the aortic sinus in HFD-fed mice. As for the percentage of the proximal aorta occupied by lesions to the total lumen, a similar tendency was seen (Fig. 1A and 1B).
1041.6 ± 591.6 mm² for the 2 μg GroEL group vs. 229.5 ± 45.9 mm² for the control group, P < 0.05; 732.2 ± 252.7 mm² for the 10 μg GroEL group vs. 229.5 ± 74.8 mm² for the control group, P < 0.05; Fig. 1A and 1C, 0.37 ± 0.10% for the 2 μg GroEL group vs. 0.11 ± 0.04% for the control group, P < 0.05; 0.33 ± 0.15% for the 10 μg GroEL group vs. 0.11 ± 0.04% for the control group, P < 0.05). However, compared to the P. gingivalis-challenged group (Fig. 1A and 1B, 7251.0 ± 524.1 mm² for P. gingivalis-challenged group vs. 3259.1 ± 270.5 mm² for the sham-challenged group, P < 0.05; Fig. 1A and 1C, 3.72 ± 0.37% for P. gingivalis-challenged group vs. 1.62 ± 0.19% for the sham-challenged group, P < 0.05), the increase was slight. In contrast, GroEL immunization did not affect plaque formation at the aortic sinus in the RD-fed mice (457.1 ± 157.8 mm² for the 10 μg GroEL group vs. 582.5 ± 114.4 mm² for the control group; 0.22 ± 0.08% for the 10 μg GroEL group vs. 0.26 ± 0.04% for the control group).

**Serum antibody levels**

Immunization of mice with GroEL plus incomplete Freund’s adjuvant produced a sufficient GroEL-specific serum IgG response in mice fed the HFD at 13 and 16 weeks (data not shown). Furthermore, prime-boost immunization induced a maximal antibody titer (Fig. 2). The difference in antigen dose was not reflected in the antibody titer. The
mean levels of anti-HSP60 antibodies were also detected in mice immunized with GroEL. Furthermore, when serum samples were incubated with HSP60, the levels of anti-GroEL antibodies were reduced compared with samples incubated with PBS, indicating a cross reactivity with HSP60. In contrast, neither GroEL-specific nor HSP60-specific serum IgG antibodies were detected in non-immunized mice and mice that inoculated i.v. with live P. gingivalis (Fig.2).

Identification of HSP60

Immunostaining of the paraffin-embedded aortic sinus sections revealed marked upregulation of HSP60 production in HFD-fed mice compared to RD-fed mice (Fig.3A). Furthermore, HFD consumption significantly increased the mRNA expression of HSP60 in aorta (P < 0.05; Fig.3B). No difference was observed between the immunized mice and controls in terms of HSP60 expression (data not shown). Furthermore, we have checked the correlation between atherosclerotic lesion area and HSP60 gene expression in GroEL-immunized mice. There was no correlation between HSP60 expression and disease severity measured by atherosclerotic plaque size (data not shown).

Serum MCP-1 and CRP levels

To further analyze the levels of systemic inflammation in the immunized animals, we measured the serum MCP-1 and CRP levels in each group of mice at 24 weeks by ELISA. Although immunization with 2 μg of GroEL slightly increased the serum MCP-1 levels in HFD-fed mice, the difference was not statistically significant between the control and 10 μg GroEL group (Fig.4). Furthermore, GroEL immunization did not affect MCP-1 levels in RD-fed mice (data not shown). The serum CRP concentrations in the GroEL-immunized groups were not significantly higher compared to the control groups in which mice were fed HFD (Fig.4) or RD (data not shown).

Aortic expression of CD40, CD40L, and LOX-1

To further analyze the influence of extracellular stress proteins in immunized animals, we measured the expression of cell-surface receptors such as CD40, CD40L, and LOX-1 in the aorta. Mice immunized with GroEL displayed a significant increase in the mRNA expression of CD40 (P < 0.05 for 10 mg of GroEL) and LOX-1 (P < 0.05) compared with the non-immunized group (Fig.5). CD40L expression was also markedly higher in mice immunized with 10 μg of GroEL than in control mice, although the difference was not statistically significant.

Discussion

Previous investigations have shown that P. gingivalis accelerates the progression of atherosclerosis in apoE-deficient mice or HFD-fed normal mice (8, 9). We hypothesized that P. gingivalis challenge induced the increase of anti-GroEL antibody followed by the acceleration of atherosclerosis. Therefore, we used P. gingivalis-challenged mice for positive control. Immunization of BALB/c mice with P. gingivalis GroEL was insufficient to accelerate atherosclerosis compared to P. gingivalis-challenged mice, although lesion area, serum levels of antibodies directed to GroEL and HSP60, and expression of cell-surface receptors, such as LOX-1 and CD40/40L in the aorta, were increased.

Several studies have indicated that HSP60 immunity is associated with atherosclerosis development (23). A significantly higher anti-hHSP60 antibody titer was found in individuals with future CVD compared with individuals without cardiovascular events (24). Furthermore, the anti-P. gingivalis GroEL antibody titers were also higher in patients with atherosclerosis compared with healthy
Fig. 3  HSP60 expression in HFD-fed mice. (A) Immunohistochemical analyses of HSP60 in aortic sinuses of mice fed either HFD or RD. Scale bars = 50 μm (×200) and 100 μm (×100). (B) Gene expression of HSP60 in the aortic sinuses of mice fed either HFD or RD. Relative mRNA levels were obtained using real-time RT-PCR after normalization to β-actin. Data are expressed as the fold increase in mRNA level compared to the RD-fed mice. The values represent the mean ± SD of six mice per group. P < 0.05 compared to the RD-fed group.

Fig. 4  Serum MCP-1 and CRP levels in GroEL-immunized mice fed a HFD. Serum samples were collected at 13, 16, and 24 weeks and assessed for MCP-1 and CRP by ELISA. The data represent the mean ± SD of six mice per group. P < 0.05, significant difference from the PBS-inoculated group.
HSP60 is conserved across species, and immune reactions to HSP60 have been widely described in infectious disease due to bacteria that express HSP60. Anti-HSP60 antibodies induced in response to these microbes can cross-react through molecular mimicry with self-HSP60 expressed on endothelial cells. Increased levels of anti-HSP60 antibodies have been associated with the severity of atherosclerosis.

However, in our study, atherosclerosis induction by P. gingivalis challenge was not correlated with a high antibody titer to GroEL because anti-GroEL antibody did not increase in P. gingivalis-challenged positive control. Furthermore, although this reached statistical significance, the atherosclerotic lesion ratio in the aortic sinus of GroEL immunized group was only one-tenth compared with P. gingivalis-challenged group. Therefore, the titer does not reflect the severity of the atherosclerosis which occurs following P. gingivalis infection. Thus, induction of arteriosclerosis in P. gingivalis-challenged mice requires elements other than a high antibody titer to GroEL. It is unknown how anti–HSP60 autoantibodies promote atherosclerosis but two potential mechanisms are suggested: induction of HSP60 expression in the vascular endothelium may play a prominent role and/or an immune response mounted against antigens on pathogenic organisms may cross-react with homologous host proteins in a form of molecular mimicry.

The cellular antigen targeted by anti–HSP60 autoantibodies is presumably located on the endothelium. Mammalian HSP60 is generally considered to be an intracellular protein expressed only on the surface of cells that have been activated or stressed. The effects of dietary constituents on HSP expression have been studied in animal models. Romano et al. reported that a diet rich in saturated fatty acids induced the expression of HSP-25, -60, and -70 in mouse splenic lymphocytes. Lamb and Fern also reported a rise in serum anti-HSP titers in rabbits fed a high-cholesterol diet. Therefore, it is conceivable that an autoimmune response via molecular mimicry between a bacterial antigen and self-antigen may be due to a combination of increased HSP expression and an enhanced immune response, both of which are associated with a diet high in saturated fats and P. gingivalis GroEL.

In this study, the antibody titer was maintained 9 weeks after the last immunization. Bacterial GroEL is regarded as the primary antigen that may cause an autoimmune response via molecular mimicry. GroEL is also an immunodominant antigen in patients with periodontitis. A positive relationship between periodontitis and the serum level of antibodies directed against GroEL has been observed. Furthermore, cross-reactivity between plasma anti–GroEL and –P. gingivalis antibodies with hHSP60 has been demonstrated in patients with atherosclerosis. Therefore, previous results suggest that increased GroEL in periodonti-
tis, even if the increase is minimal, may play an important role in the initiation of atherosclerosis. In this study, we have also observed the cross-reactivity of serum anti-GroEL with HSP60 in GroEL-immunized mice. Therefore, the cross-reactive immune response may cause slight atherosclerotic plaque formation in GroEL-immunized mice.

We demonstrated that GroEL upregulated LOX-1 and CD40/40L expression in the aorta of HFD-fed mice. Recent studies have demonstrated that LOX-1 is a multiligand receptor that also binds to CRP, apoptotic cells, bacteria, and HSPs (33). Furthermore, LOX-1 activation induces CD40 expression/CD40L signaling in human coronary artery endothelial cells (34). CD40/40L is related to plaque instability and vascular inflammation (35, 36). Therefore, not the GroEL antibody, but rather HSP60 alone may facilitate LOX-1 expression by binding to LOX-1, followed by vascular inflammation and endothelial dysfunction resulting in atherosclerosis.

In conclusion, our results demonstrate that antibody responses to P. gingivalis GroEL are not enough to cause P. gingivalis–driven atherosclerosis, although GroEL immunization in the aorta of HFD–fed BALB/c mice results in a cross reactivity with HSP60 and a slight secretion of serum MCP-1 and LOX-1 and CD40/40L expression. These results suggest a different mechanism of autoimmune reactions for the acceleration of atherosclerosis in periodontal infection.

Acknowledgments
This study was supported by Grants-in-Aid for Scientific Research (22300398) from the Japan Society for the Promotion of Science, and by the “Strategic Research Base Development” Program (Japan [MEXT], 2010–2014 [S1001024]) for Private Universities of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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