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Original Article

Oral immunization with *Porphyromonas gingivalis* outer membrane protein and CpG oligodeoxynucleotides attenuates *P. gingivalis*-accelerated atherosclerosis and inflammation

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ABSTRACT

Objective: It has previously been shown that oral immunization with the 40-kDa outer membrane protein of *Porphyromonas gingivalis* (40k-OMP) and CpG oligodeoxynucleotides (ODN) as an adjuvant elicits protective antibody responses against alveolar bone loss caused by *P. gingivalis* infection. The objective of the present work was to assess the efficacy of this same oral vaccine on prevention of *P. gingivalis*-accelerated atherosclerosis.

Methods: Apolipoprotein E-deficient spontaneously hyperlipidemic (Apoe^{shl}) mice were orally immunized with 40k-OMP plus CpG ODN and subsequently challenged intravenously with *P. gingivalis*. The mice were euthanized 15 weeks later, and atheromatous lesions in the proximal aorta of each mouse were analyzed histomorphometrically. Serum concentrations of 40k-OMP-specific antibodies and cytokines as well as levels of proatherogenic factors in the aorta were determined.

Results: P. gingivalis challenge resulted in an increase in the areas of the aortic sinus covered with atherosclerotic plaque, as well as in the levels of high-sensitive C-reactive protein (hsCRP) and some cytokines and chemokines, when compared with sham-treated mice. In contrast, oral immunization with 40k-OMP plus CpG ODN induced 40k-OMP-specific serum IgG responses, and significantly reduced atherosclerotic plaque accumulation in the aortic sinus, along with hsCRP and the cytokine and chemokine levels.

Conclusions: These results suggest that oral administration of 40k-OMP plus CpG ODN may be an effective vaccine for the prevention of accelerated atherosclerosis caused by *P. gingivalis* infection.

1. Introduction

The incidence of atherosclerotic cardiovascular disease is increasing, and has become a leading cause of death [1]. Emerging evidence suggests that infection with specific pathogens is an additional risk factor for atherosclerosis [2]. In this regard, previous studies have shown that periodontitis is associated with endothelial dysfunction [3], atherosclerosis [4], and an increased risk of myocardial infarction [5]. It has been shown that periodontal disease pathogens reside in the walls of atherosclerotic vessels [6]. In addition, DNA from periodontal pathogens, including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, have been detected in atheromatous plaques [6,7].

The 40-kDa outer membrane protein (40k-OMP) gene was originally cloned from *P. gingivalis* FDC381 into *Escherichia coli*. The resulting clone, designated MD123, contained plasmid pMD123 with a 2.0 kbp insert from *P. gingivalis*, and expressed a protein with an apparent molecular mass of 40 kDa [8]. 40k-OMP is a key virulence factor for coaggregation and hemagglutination [9,10]. We showed previously that nasal administration of the 40k-OMP with cholera toxin (CT) as an adjuvant provided protection against *P. gingivalis* infection [11]. Furthermore, when apolipoprotein E-deficient spontaneously hyperlipidemic mice (Apoe^{shl}) were nasally immunized with 40k-OMP plus CT prior to infection, atherosclerotic plaque accumulation in the aortic sinus was significantly reduced [12]. These studies indicate that 40k-OMP could be an effective vaccine antigen (Ag) for the prevention of *P. gingivalis* infection.

Oral-gastric delivery of vaccines is a preferred route of immunization and is usually called oral immunization. It offers several advantages over other Ag delivery systems. However, mucosal vaccines, including oral vaccines, generally require the use of

Abbreviations: 40k-OMP, 40-kDa outer membrane protein of *Porphyromonas gingivalis*; CpG ODN, synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides; CT, cholera toxin

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adjuvants to enhance specific immunity [13]. Bacterial toxins, such as CT, are commonly used as mucosal adjuvants in animal models; however, toxicity prevents their use in humans [14]. Genetically detoxified CT mutants have been developed by site-directed mutagenesis and appear to be non-toxic in animal models while retaining adjuvanticity [15]. Despite this progress, there remains a need for novel safe and effective mucosal adjuvants. A new adjuvant class includes synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG motifs). CpG ODN interacts with toll like receptor (TLR)-9 expressed by B cells and dendritic cells, and induces Type 1T helper (Th1) cell and proinflammatory cytokine responses [16]. A number of immunization studies have reported that parenteral immunization of animals with various Ags plus CpG ODN as an adjuvant induces Th1-type responses, as indicated by high levels of IgG2a antibodies (Abs) and Th1 cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ [17]. Furthermore, it has been shown that CpG ODN is a potent adjuvant when given nasally [18] or orally [19]. Our previous study also indicated that oral administration of 40k-OMP together with CpG ODN induced Th1- and Th2-type immune responses in mice [20].

Apoe^{shl} mice, an inbred strain created from Japanese wild mice, are deficient in apoE expression due to a gross disruption of the *apoE* gene [21]. These mice show hypercholesterolemia and accumulate large amounts of remnant-like particles in the bloodstream, as has been observed in *apoE* knockout mice [12]. In this study, we used congenic mice with a BALB/c genetic background as an alternative animal model of apolipoprotein E-deficiency to examine the effect of oral 40k-OMP plus CpG ODN on atherosclerosis accelerated by *P. gingivalis* infection.

2. Materials and methods

2.1. Bacterial strain and injection

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% H_2, 80% N_2, and 10% CO_2 for 3–5 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 at 37 °C for 2 days until they reached an optical density of 0.8 at 660 nm, corresponding to 10^9 CFU/mL. The cultured cells were then centrifuged at 8000 × *g* for 15 min at 4 °C and diluted with phosphate-buffered saline (PBS) for intravenous (i.v.) infection. The first group of mice was challenged with 0.1 mL of PBS by i.v. injection 3 times per week for 3 weeks, whereas the second group was challenged with 0.1 mL of live *P. gingivalis* (10^8 CFU/mouse) by i.v. injection 3 times per week for 3 weeks (Fig. 1). The third and fourth groups were orally immunized with 40k-OMP plus CpG ODN and 40k-OMP alone in sterile, pyrogen-free PBS, respectively, once a week for 3 weeks, prior to *P. gingivalis* challenge (Fig. 1).

2.2. Antigen and adjuvant

Plasmid pMD125 expressing 40k-OMP was kindly provided by Dr. Yoshimitsu Abiko from Nihon University. The 40k-OMP protein was purified from a cell suspension of *E. coli* K-12 harboring pMD125, as described previously [22]. The purity of 40k-OMP was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and no contaminating protein bands were noted. Furthermore, possible residual endotoxin was assessed in the preparation with a limulus amebocyte lysate (LAL) pyrochrome kit (Associates of Cape Cod, Inc., Woods Hole, MA). One milligram of the 40k-OMP preparation contained as little as 0.3 pg of endotoxin. CpG ODNs (5′-TCCATGACGTTCCTGACGTT-3′) were purchased from Coley Pharmaceutical Group, Inc. (Wellesley, MA).

2.3. Animals

All experiments were performed using 8-week-old female BALB/c, apoE-deficient spontaneously hyperlipidemic (c.KOR-Apoe^{shl}) mice, which were purchased from Sankyo Lab Services (Tokyo, Japan) and were maintained in our experimental facility under pathogen-free conditions. The institutional Animal Care and Use Committee of Nihon University approved all animal protocols. Mice were given regular mouse chow and water, and were randomly divided into 3 groups ($n = 8$ for each group; Fig. 1). All mice were monitored daily until sacrifice and appeared healthy throughout the course of the study. Mice from each group were euthanized at 15 weeks of age, and tissues and blood samples were collected.

2.4. Immunization and sample collection

The immunization groups were primed on Day 0 and boosted on Days 7 and 14. Before immunization, each mouse was deprived of food for 2 h and then given an isotonic solution (250 μL). After 30 min, mice were orally immunized with 200 μL of PBS containing 200 μg of 40k-OMP alone or in combination with 10 μg of

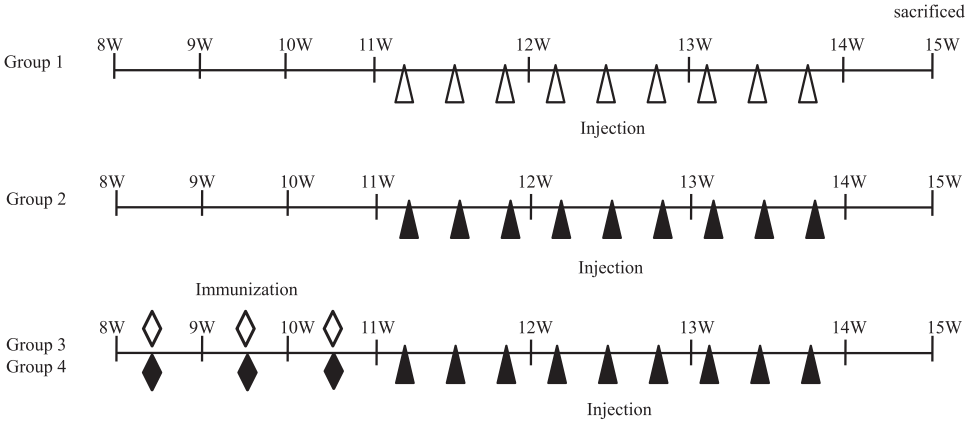

Fig. 1. Experimental procedure. Eight-week-old female Apoe^{shl} mice were randomly divided into 3 groups: Group 1 was inoculated with 100 μL of PBS (▵), Group 2 was inoculated with 100 μL (10^8 CFU) of *P. gingivalis* (▲), Group 3 was immunized with 40k-OMP plus CpG ODN (◇) and inoculated with 100 μL (10^8 CFU) of *P. gingivalis* (▲), and Group 4 was immunized with 40k-OMP (◆) and inoculated with 100 μL (10^8 CFU) of *P. gingivalis* (▲). The immunized mice were orally vaccinated with 40k-OMP plus CpG ODN once per week for 3 weeks prior to the bacterial challenge. Mice were i.v. challenged with *P. gingivalis* strain 381 three times per week for 3 weeks. Mice were sacrificed 1 week after the final challenge. W, week.

CT or 500 μg of CpG ODN. Serum was collected from each group to examine specific Ab responses.

2.5. 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) [12]. The heart and aortic tree were then perfused through the left ventricle with iced 0.9% PBS for 10 min. The heart was carefully dissected and removed. The upper half of the heart containing the aortic origin was separated and embedded in Tissue-Tek® OCT compound in cryomolds (Fisher Scientific, Newark, DE), and cryostat sections were prepared [23]. Using a modified version of the method of Paigen et al. [23], we examined the cryosections of the aortic sinus for atherosclerotic plaque accumulation using oil red-O staining. The lesion area was then quantified using a microscope interfaced with a charge-coupled device camera and an image analysis system (BX51; Olympus, Tokyo, Japan). Briefly, cross-sectional areas from 3 images were added to obtain the total lesion area per slide, and the percentage of the aortic lumen that was occupied by lesions per section was calculated. Slide analysis was conducted in a blinded fashion. Finally, the percentage of the aortic lumen occupied by lesions was averaged over 15 sections per animal and expressed as the percentage of the lumen of the proximal aorta occupied by lesions per section per animal.

2.6. Detection of 40k-OMP-specific IgG, CRP, and cholesterol levels in serum

Ab titers were determined using enzyme-linked immunosorbent assays (ELISAs) [24]. Briefly, plates were coated with 40k-OMP (5 μg/mL) and blocked with PBS containing 1% bovine serum albumin. After blocking, serial dilutions of serum samples were performed in duplicate. The starting dilution of serum was $1{:}2^5$. After 4 h of incubation at room temperature, the plates were washed and goat horseradish peroxidase (HRP)-conjugated anti-mouse γ heavy chain-specific Ab (Southern Biotechnology Associates, Birmingham, AL) was added to the appropriate wells. Finally, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) with H_2O_2 (Moss, Inc., Pasadena, MD) was added for color development. The endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 415 nm of 0.1 units greater than the non-immunized control samples after 15 min of incubation. The serum samples from blood collected at sacrifice were also screened by ELISA for hsCRP (Kamiya Biomedical Company, WA). The serum levels of total cholesterol were determined using a high-density lipoprotein (HDL) and low-density lipoprotein (LDL)/very low-density lipoprotein (VLDL) cholesterol quantitative kit (BioVision, Mountain View, CA).

2.7. Cytokine Ab array

Serum samples from two mice per group were pooled and analyzed using a cytokine Ab array (RayBio Mouse Atherosclerosis Ab array I; Ray Biotech, Inc., Norcross, GA) comprising 22 different atherosclerosis-related cytokine Abs, which were spotted in duplicate on a membrane. Briefly, the cytokine array membranes were blocked in 2 mL of 1 × blocking buffer for 30 min and then incubated with 1 mL of sample at room temperature for 1–2 h. The samples were then decanted from each container, and the membranes were washed 3 times with 2 mL of 1 × Wash Buffer I, followed by 2 washes with 2 mL of 1 × Wash Buffer II at room temperature on a shaker. The membranes were then incubated in a 1:250 dilution of biotin-conjugated primary Ab at room temperature for 1–2 h and then washed as described above prior to incubation in a 1:1000 dilution of HRP-conjugated streptavidin. After incubation for 30–60 min, the membranes were thoroughly washed and exposed to a peroxide substrate (Detection Buffers C and D; Ray Biotech, Inc., Norcross, GA) for 5 min in the dark before imaging. Chemiluminescent signals from the bound cytokines were detected by using the Lumino Image Analyzer (Fuji Film, Tokyo, Japan). The signal intensities were quantified using an Image Reader LAS-1000 (Fuji Film) and analyzed using Image Gauge (Fuji Film). Biotin-conjugated IgG served as a positive control in 6 spots, where it was used to identify the membrane orientation and to normalize the results from different membranes. For each spot, the net optical density was determined by subtracting the background optical level from the total raw optical density. The level of each cytokine was represented as a percentage of the positive control.

2.8. Real-time quantitative reverse transcription (RT)-PCR

Purified total RNA obtained from each aorta ($n = 8$ in each group) was reverse transcribed with Oligo (dT) using SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, CA) to generate cDNA. Real-time quantitative RT-PCR analyses were performed using the Thermal Cycler Dice™ Real Time System (Takara, Shiga, Japan) in accordance with the manufacturer's protocol. The reactions contained 30 ng of reverse-transcribed RNA, 12.5 μL of 2 × SYBR Green PCR Master Mix (Takara), and each primer at 100 nM (final volume, 25 μL). The program included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, at 55 °C for 25 s, and at 60 °C for 35 s. Each gene was tested in triplicate. The primers used are shown in Table 1. The amount of RNA was normalized relative to GAPDH mRNA levels.

2.9. Histology and morphometric analysis

The heart, kidneys, spleen, liver, and small intestine tissues were fixed in 10% neutral buffered formalin for 24 h. Samples were processed and embedded for paraffin sectioning to obtain sections of 5 μm in thickness. All sections were stained with hematoxylin and eosin for structural observation.

Table 1
Primers sequences used for real-time RT-PCR.

Primers	Forward	Reverse
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
HSP60	TGCTAAGAATGCAGGTGTTGAAGG	AGCTTCGGCTGTAGTTAGCAAGGA
TLR4	GGGCCTAAACCCAGTCTGTTTG	GCCCGGTAAGGTCCATGTA
VCAM-1	TGCCGGCATATACGAGTGTGA	CCCGATGGCAGGTATTACCAAG
CCL21	ACTTGCGGCTGTCCATCTCA	AGCCTCGGACAATACTGTAGGGAAT
MMP2	GATAACCTGGATGCCGTCGTG	CTTCACGCTCTTGAGACTTTGGTTC
CCR7	TGGTCAGTGCCCAAGTGGAG	TCAAAGTTGCGTGCCTGGAG
ICAM-1	CAATTCACACGAATGCCAGCTC	CAAGCAGTCCGTCTCGTCCA

2.10. Statistical analysis

The data are presented as the mean ± standard deviation (SD). Differences in the mean values among groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Health assessment

No clinical signs of infection or mortality were noted in any of the animals at any time. There were no significant differences in body weight between *P. gingivalis*- and sham-inoculated mice. The heart, kidneys, spleen, liver, and small intestine of all animals showed normal histological structure.

3.2. Effect of oral immunization on P. gingivalis-specific IgG and serum cholesterol levels

In order to evaluate the ability of oral immunization with 40k-OMP to induce serum antibody responses, a group of mice was orally immunized with 40k-OMP alone. 40k-OMP-specific IgG antibody responses were detected; however, the responses were low (data not shown). Immunization of mice with 40k-OMP plus CpG ODN produced a maximal 40k-OMP-specific serum IgG response compared to the other groups (Fig. 2). In contrast, negligible amounts of 40k-OMP-specific serum IgG Abs were produced by the non-immunized mice (Fig. 2) or by the mice that received the adjuvant alone (data not shown). The sham-inoculated hyperlipidemic mice had high total cholesterol (1930 ± 80 mg/dL), LDL (1610 ± 10 mg/dL), and HDL (350 ± 120 mg/dL) levels (Fig. 3); whereas i.v. challenge with *P. gingivalis* or immunization had no effect on the levels of these molecules (total cholesterol: 2250 ± 150 mg/dL for *P. gingivalis* challenge, and 2100 ± 240 mg/dL for immunization; LDL: 1720 ± 130 mg/dL for *P. gingivalis* challenge, and 1720 ± 30 mg/dL for immunization; and HDL: 530 ± 50 mg/dL for *P. gingivalis* challenge, and 380 ± 270 mg/dL for immunization; Fig. 3).

3.3. Oral immunization with 40k-OMP plus CpG ODN prevents P. gingivalis-accelerated atherosclerosis

Histomorphological analysis showed that *P. gingivalis* inoculation markedly increased atherosclerotic plaque accumulation in Apoe^{shl} mice compared with sham-treated mice (8.3 ± 0.17% for *P. gingivalis* vs. 2.2 ± 1.2% for PBS, $P < 0.01$; Fig. 4). In contrast, oral immunization with 40k-OMP plus CpG ODN reduced atherosclerotic plaque accumulation in the *P. gingivalis*-infected group at 15 weeks (8.3 ± 0.17% for *P. gingivalis* vs. 2.8 ± 1.8% for 40k-OMP + CpG ODN + *P. gingivalis*, $P < 0.05$; Fig. 4). Oral immunization with CpG ODN alone did not reduce atherosclerotic plaque accumulation in the *P. gingivalis*-challenge group (data not shown). Serum hsCRP concentrations were higher in *P. gingivalis*-challenged mice compared with the controls (11.1 ± 1.3 vs. 0.4 ± 0.01 ng/mL, $P < 0.001$; Fig. 5). In contrast, the rise in hsCRP concentration caused by *P. gingivalis* challenge was significantly reduced in mice immunized with 40k-OMP plus CpG ODN.

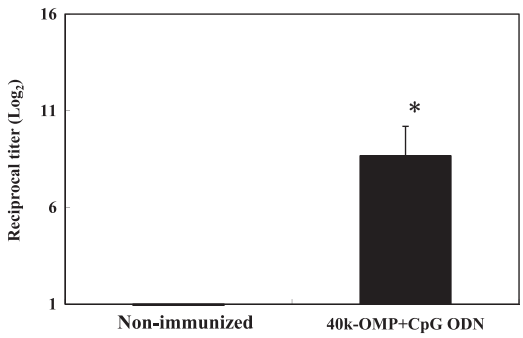

Fig. 2. 40k-OMP-specific Ab response after oral vaccination with 40k-OMP plus CpG ODN. Apoe^{shl} mice were orally immunized with 10 mg of 40k-OMP plus 500 μg of CpG ODN (■) or were not immunized (□). Serum samples were collected on Day 21 and assessed for 40k-OMP-specific serum IgG Abs. The results are expressed as the mean ± SD of 6 mice per group. $^{*}P < 0.05$ vs. the non-immunized group.

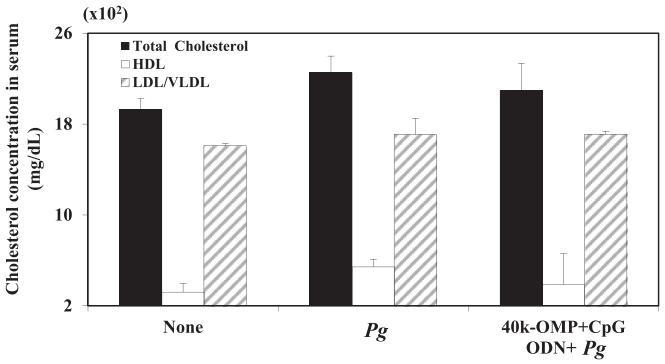

Fig. 3. Serum cholesterol and CRP levels after *P. gingivalis* challenge and/or oral vaccination with 40k-OMP plus CpG ODN. Serum levels of (A) total (■), LDL (▨), and HDL cholesterol (□), and of (B) CRP, were examined using a quantitative kit. The results are expressed as the mean ± SD of 6 mice per group. *Pg*, *P. gingivalis*.

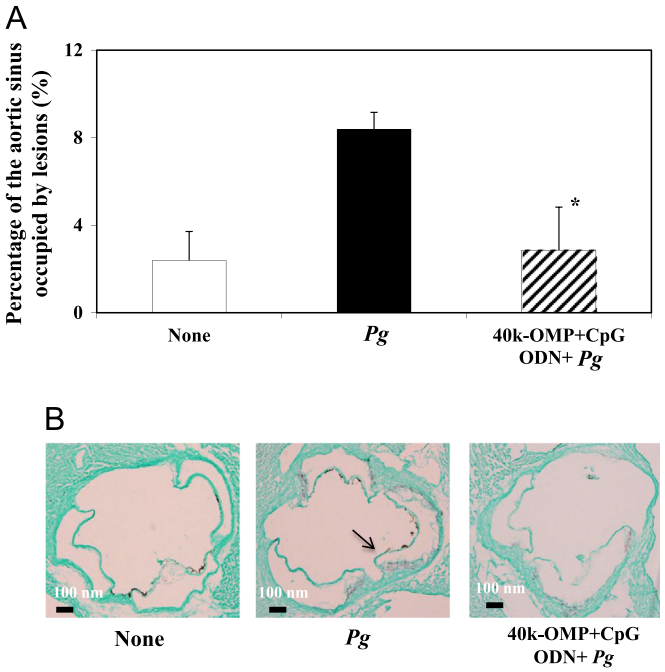

Fig. 4. Atherosclerotic plaque formation in the aortic sinuses of Apoe^{shl} mice challenged intravenously with *P gingivalis*. Histomorphometric analyses indicating the percentage of the aortic sinuses occupied by lesions at 15 weeks are shown. The results are expressed as the mean ± SD of 6 mice per group. $^{**}P < 0.01$ vs. control mice. *Pg*, *P. gingivalis*.

3.4. Cytokine array

Cytokine array analysis showed that *P. gingivalis* challenge had a significant inductive effect on the levels of serum cytokines in

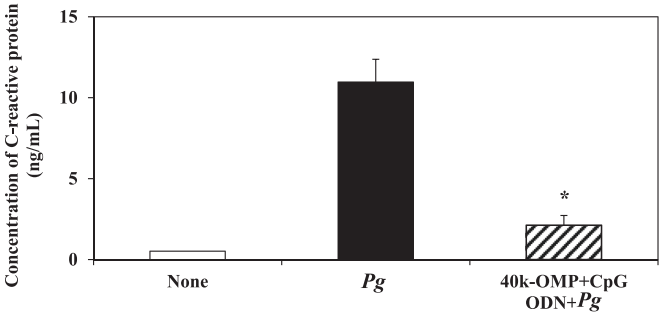

Fig. 5. Serum CRP levels after *P. gingivalis* challenge and/or oral vaccination with 40k-OMP plus CpG ODN. Serum levels of (A) total (■), LDL (▨), and HDL cholesterol (□), and of (B) CRP were examined using a quantitative kit. The results are expressed as the mean ± SD of 6 mice per group. *Pg*, *P. gingivalis*.

mice; in other words, all cytokine levels examined were significantly increased by *P. gingivalis* challenge. On the other hand, among the cytokines examined, the increase in IL-1α ($P < 0.01$), IL-2 ($P < 0.05$), monocyte chemoattractant protein-1 (MCP-1; $P < 0.05$), macrophage colony stimulating factor (M-CSF; $P < 0.05$), P-selectin ($P < 0.05$), and RANTES (for regulated on activation, normal T-cell expressed and secreted) levels ($P < 0.01$) was significantly lower in mice immunized with 40k-OMP plus CpG ODN than in mice challenged with *P. gingivalis* alone (Fig. 6). Conversely, IL-5 was significantly increased in immunized mice compared to non-immunized challenged mice ($P < 0.05$).

3.5. Detection of proatherogenic factors by real-time PCR

To examine the involvement of various inflammatory mediators in atherosclerosis enhanced by *P. gingivalis* challenge, we analyzed mRNA expression in the thoracoabdominal aorta using real-time RT-PCR (Fig. 7). In the aorta, *P. gingivalis* induced a significant increase in mRNA expression of the heat shock protein 60 (HSP60; $P < 0.01$), TLR4 ($P < 0.01$), vascular cell adhesion protein 1 (VCAM-1; $P < 0.01$), chemokine (C-C motif) ligand 21 (CCL21; $P < 0.01$), and matrix metalloproteinase 2 (MMP2; $P < 0.01$). In contrast, mRNA expression of the C-C chemokine receptor type 7 (CCR7) and intercellular adhesion molecule 1 (ICAM-1) was not increased. Notably, the rise in mRNA expression of HSP60 ($P < 0.05$), TLR4 ($P < 0.01$), VCAM-1 ($P < 0.01$), CCL21 ($P < 0.01$), and MMP2 ($P < 0.01$) caused by *P. gingivalis* challenge was substantially reduced in mice immunized with 40k-OMP plus CpG ODN (Fig. 7).

4. Discussion

The present study was designed to test whether a systemic intravascular challenge with an established periodontal pathogen contributes to the development and progression of atherosclerosis in a susceptible animal model. This mode of challenge is reasonable because patients with periodontitis are thought to be chronically exposed to non-symptomatic bacteremias, the level, duration, and microbial diversity of which increase with periodontal disease severity [25]. Surgical treatment, tooth brushing, and other dental procedures can seed oral bacteria into systemic circulation [26,27]. In this study, we found that infection of atherosclerosis-prone hyperlipidemic Apoe^{shl} mice with *P. gingivalis* resulted in accelerated atherosclerosis associated with an increased plaque lipid content, elevated serum hsCRP, serum cytokine, and chemokine levels, and increased expression of TLR4, VCAM-1, CCL21, MMP-2, and HSP60 in the aorta compared with mock-infected controls. Challenge with *P. gingivalis* did not affect the serum cholesterol levels (total cholesterol, LDL, or HDL) of hyperlipidemic mice. Previous data, which compared the phenotype of Apoe^{shl} mice with that of ApoE-knockout (ApoE-KO) mice, indicated that although the serum cholesterol value of Apoe^{shl} mice was 1.5-fold higher than that of ApoE-KO mice, atherosclerotic lesions in Apoe^{shl} mice were 3–5 fold smaller than those of ApoE-KO mice [28]. Therefore, atherosclerosis progression did not correlate with serum cholesterol values. *P. gingivalis* infection might be related to the promotion of the atherogenic process regardless of plasma cholesterol-related advancement of atherosclerosis.

A recent report has shown that periodontitis is a risk factor for cardiovascular disease (CVD), even though no significant differences in cholesterol levels were detected between patients with CVD who had periodontitis and patients with CVD who did not have periodontitis [29]. On the other hand, previous observations suggested that inflammation caused by periodontopathic bacteria may play a synergistic role with other preexisting factors, such as hyperlipidemia, resulting in the development of atherosclerosis [30]. These observations support the hypothesis that periodontal pathogens are not an independent risk factor, but rather act in concert with hyperlipidemia to exacerbate atherosclerosis lesion formation.

In the present work, oral immunization with 40k-OMP plus CpG ODN protected mice against *P. gingivalis*-accelerated atherosclerosis. A recent study had shown that *P. gingivalis* is promptly eliminated from the blood by nasal immunization with 40k-OMP [12]. Other studies have also shown that a monoclonal antibody (MAb) to 40k-OMP inhibits coaggregation of *P. gingivalis* and promotes complement-mediated bactericidal and opsonic activity for the phagocytosis of *P. gingivalis* [9,10,31]. Furthermore, rats transcutaneously immunized with a 40k-OMP vaccine had significantly diminished *P. gingivalis*-induced abscess formation [12]. *P. gingivalis* is a causative agent of chronic periodontitis, which is associated with several systemic diseases, such as CVD [32–34], aspiration pneumonia [35], preterm delivery of low-birth weight fetuses [36,37], and diabetes mellitus [38,39]. Recent evidence suggests that this bacterium contributes to periodontitis by functioning as a keystone pathogen [40]. Therefore, the development of a mucosal 40k-OMP vaccine for humans may be a significant milestone in the search for an effective vaccine against *P. gingivalis* infection.

We further explored the potential benefits of 40k-OMP by studying its protective efficacy against the systemic inflammatory response initiated and/or exacerbated by *P. gingivalis*. Oral immunization with 40k-OMP induced a significant 40k-OMP-specific IgG Ab response in serum. We had previously demonstrated that oral administration of 40k-OMP plus CpG ODN as adjuvant induces a significant 40k-OMP-specific IgA antibody response in saliva [20]. Serum-derived IgG Abs are present in the clavicular fluid, which continuously flows from the gingival capillaries and is part of the systemic immune system [41]. Because *P. gingivalis* colonizes subgingival biofilms [42,43], generation of serum-derived IgG Abs in the clavicular fluid may be more effective than salivary IgA in preventing *P. gingivalis* infection. Therefore, the 40k-OMP-specific serum IgG response [20] induced by the oral vaccine might have a protective effect against *P. gingivalis*-accelerated atherosclerosis.

Atherosclerosis has a strong inflammatory component, and epidemiological evidence suggests that increased levels of systemic inflammation are predictive of cardiovascular events [44]. Our results showed an increase in hsCRP and all cytokine levels examined in the serum of mice receiving live *P. gingivalis* compared with mock-challenged controls. However, the levels of hsCRP and cytokines were reduced in the immunized mice. Specifically, a significant decrease in IL-1α ($P < 0.01$), IL-2 ($P < 0.05$), MCP-1 ($P < 0.05$), M-CSF ($P < 0.05$), P-selectin ($P < 0.05$), and RANTES ($P < 0.01$) levels was observed in mice immunized with

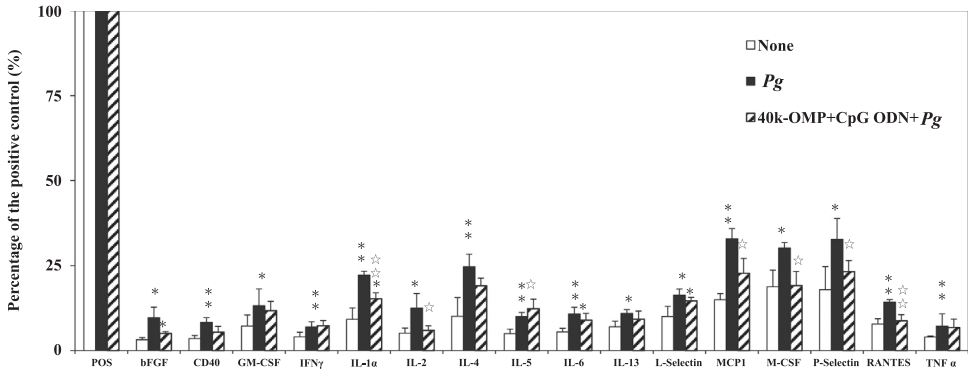

Fig. 6. Cytokine profiles from serum samples of mice challenged with *P. gingivalis* or oral immunized, collected at 15 weeks. The level of each cytokine is presented as a percentage of the positive control. The data represent the mean ± SD of three independent experiments of 3 pooled samples. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. PBS-treated control mice; $^{☆}P < 0.05$, $^{☆☆}P < 0.01$ vs. *P. gingivalis*-challenged mice. POS, positive control; βFGF, beta fibroblast growth factor; CD40, cluster of differentiation 40; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; TNF-α, tumor necrosis factor; *Pg*, *P. gingivalis*.

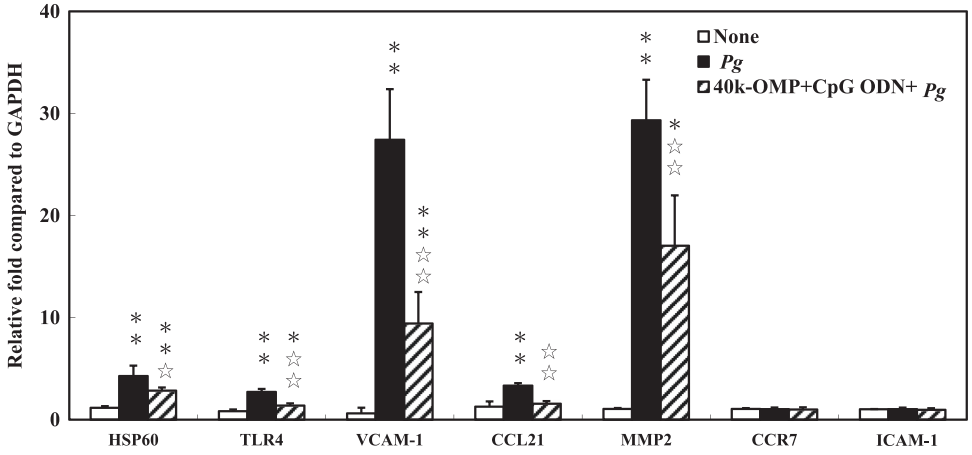

Fig. 7. Proatherogenic gene expression in response to *P. gingivalis* challenge or oral immunization with 40k-OMP plus CpG ODN in the aorta of Apoe^{shl} mice at 15 weeks. Relative mRNA levels normalized to GAPDH were determined by real-time reverse transcription polymerase chain reaction. All values represent the mean ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. PBS-treated control mice; $^{☆}P < 0.05$, $^{☆☆}P < 0.01$ vs. *P. gingivalis*-challenged mice. HSP60, heat shock protein 60; TLR4, toll-like receptor 4; VCAM-1, vascular cell adhesion molecule-1; CCL21, chemokine (C-C motif) ligand 21; MMP2, matrix metalloproteinase-2; CCR7, C-C chemokine receptor type 7; ICAM-1, intercellular adhesion molecule-1; *Pg*, *P. gingivalis*.

40k-OMP plus CpG ODN. On the other hand, IL-5 levels increased in the immunized group. This could be due to an increase in secretory IgA by the mucosal immune response, although secretory IgA were not measured in this study. *P. gingivalis* infection also upregulated the aortic expression of several other inflammatory molecules, including HSP60, TLR4, VCAM-1, CCL21, and MMP2, which have significant roles in the initiation and/or acceleration of atherosclerosis. In contrast, oral immunization with 40k-OMP plus CpG ODN significantly decreased the expression of these molecules. Therefore, these data suggest the possibility that oral immunization with 40k-OMP plus CpG ODN may control the progression of inflammation induced by *P. gingivalis*.

Finally, although several possible mechanisms may be involved in acceleration of atherosclerosis by *P. gingivalis* [45], prevention of *P. gingivalis* infection may be an effective way to reduce induction of *P. gingivalis*-related atherosclerosis, in addition to periodontitis. Therefore, preventing periodontitis might be relevant not only for oral health but also for systemic health. In conclusion, our results suggest that oral immunization with 40k-OMP plus CpG ODN provides a foundation for and warrants further research in humans, as it may lead to the development of effective and safe vaccines against *P. gingivalis* infection.

5. Conclusion

Oral immunization with 40k-OMP plus CpG-ODN significantly reduced *P. gingivalis*-induced atherosclerotic plaque accumulation in the aortic sinus. In addition, hsCRP and certain cytokine and chemokine levels increased by *P. gingivalis* infection were also significantly reduced by oral immunization with 40 k-OMP plus CpG-ODN. These results suggest that oral immunization with a periodontal antigen and CpG-ODN constitutes an effective and safe vaccine for preventing *P. gingivalis*- accelerated atherosclerosis.

Ethics approval

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of Nihon University.

Conflict of interest

The authors declare no conflicts of interest.

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References

[1] Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk-factors: global Burden of Disease Study. Lancet 1997;349:1436–42.
[2] Li L, Messas E, Batista Jr. EL, Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation 2002;105:861–7.
[3] Amar S, Gokce N, Morgan S, Loukideli M, Van Dyke TE, Vita JA. Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. Arterioscler Thromb Vasc Biol 2003;23:1245–9.
[4] Beck JD, Elter JR, Heiss G, Couper D, Mauriello SM, Offenbacher S. Relationship of periodontal disease to carotid artery intima-media wall thickness: the atherosclerosis risk in communities (ARIC) study. Arterioscler Thromb Vasc Biol 2001;21:1816–22.
[5] Scannapieco FA, Bush RB, Paju S. Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease, and stroke. A systematic review. Ann Periodontol 2003;8:38–53.
[6] Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. J Periodontol 2000;71:1554–60.
[7] Kozarov EV, Dorn BR, Shelburne CE, Dunn Jr. WA, Progulske-Fox A. Human atherosclerotic plaque contains viable invasive Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Arterioscler Thromb Vasc Biol 2005;25:e17–8.
[8] Abiko Y, Hayakawa M, Aoki H, Kikuchi T, Shimatake H, Takiguchi H. Cloning of a Bacteroides gingivalis outer membrane protein gene in Escherichia coli. Arch Oral Biol 1990;35:689–95.
[9] Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H. Role of Porphyromonas gingivalis 40-kDa outer membrane protein in the aggregation of P. gingivalis vesicles and Actinomyces viscosus. Arch Oral Biol 1992;37:717–24.
[10] Saito S, Hiratsuka K, Hayakawa M, Takiguchi H, Abiko Y. Inhibition of a Porphyromonas gingivalis colonizing factor between Actinomyces viscosus ATCC 19246 by monoclonal antibodies against recombinant 40-kDa outer-membrane protein. Gen Pharmacol 1997;28:675–80.
[11] Momoi F, Hashizume T, Kurita-Ochiai T, Yuki Y, Kiyono H, Yamamoto M. Nasal vaccination with the 40-kilodalton outer membrane protein of Porphyromonas gingivalis and a nontoxic chimeric enterotoxin adjuvant induces long-term protective immunity with reduced levels of immunoglobulin E antibodies. Infect Immun 2008;76:2777–84.
[12] Koizumi Y, Kurita-Ochiai T, Oguchi S, Yamamoto M. Nasal immunization with Porphyromonas gingivalis outer membrane protein decreases P. gingivalis-induced atherosclerosis and inflammation in spontaneously hyperlipidemic mice. Infect Immun 2008;76:2958–65.
[13] Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 2003;21(Suppl 2):S89–95.
[14] Spangler BD. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol Rev 1992;56:622–47.
[15] Yamamoto M, McGhee JR, Hagiwara Y, Otake S, Kiyono H. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. Scand J Immunol 2001;53:211–7.
[16] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.
[17] Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997;3:849–54.
[18] McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol 1998;161:4463–6.
[19] McCluskie MJ, Weeratna RD, Krieg AM, Davis HL. CpG DNA is an effective oral adjuvant to protein antigens in mice. Vaccine 2000;19:950–7.
[20] Liu C, Hashizume T, Kurita-Ochiai T, Fujihashi K, Yamamoto M. Oral immunization with Porphyromonas gingivalis outer membrane protein and CpG oligodeoxynucleotides elicits T helper 1 and 2 cytokines for enhanced protective immunity. Mol Oral Microbiol 2010;25:178–89.
[21] Matsushima Y, Hayashi S, Tachibana M. Spontaneously hyperlipidemic (SHL) mice: japanese wild mice with apolipoprotein E deficiency. Mamm Genome 1999;10:352–7.
[22] Kawamoto Y, Hayakawa M, Abiko Y. Purification and immunochemical characterization of a recombinant outer membrane protein from Bacteroides gingivalis. Int J Biochem 1991;23:1053–61.
[23] Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231–40.
[24] Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J Immunol 1998;161:4115–21.
[25] Mattila KJ, Valtonen VV, Nieminen MS, Asikainen S. Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. Clin Infect Dis 1998;26:719–34.
[26] Dorn BR, Dunn Jr. WA, Progulske-Fox A. Invasion of human coronary artery cells by periodontal pathogens. Infect Immun 1999;67:5792–8.
[27] Daly CG, Mitchell DH, Highfield JE, Grossberg DE, Stewart D. Bacteremia due to periodontal probing: a clinical and microbiological investigation. J Periodontol 2001;72:210–4.
[28] Matsushima Y, Sekine T, Kondo Y, Sakurai T, Kameo K, Tachibana M, et al. Effects of taurine on serum cholesterol levels and development of atherosclerosis in spontaneously hyperlipidaemic mice. Clin Exp Pharmacol Physiol 2003;30:295–9.
[29] Higashi Y, Goto C, Hidaka T, Soga J, Nakamura S, Fujii Y, et al. Oral infection-inflammatory pathway, periodontitis, is a risk factor for endothelial dysfunction in patients with coronary artery disease. Atherosclerosis 2009;206:604–10.
[30] Fukasawa A, Kurita-Ochiai T, Hashizume T, Kobayashi R, Akimoto Y, Yamamoto M. Porphyromonas gingivalis accelerates atherosclerosis in C57BL/6 mice fed a high-fat diet. Immunopharmacol Immunotoxicol 2012;34:470–6.
[31] Abiko Y, Ogura N, Matsuda U, Yanagi K, Takiguchi H. A human monoclonal antibody which inhibits the coaggregation activity of Porphyromonas gingivalis. Infect Immun 1997;65:3966–9.
[32] Iwai T, Inoue Y, Umeda M, Huang Y, Kurihara N, Koike M, et al. Oral bacteria in the occluded arteries of patients with Buerger disease. J Vasc Surg 2005;42:107–15.
[33] Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K. Correlation between detection rates of periodontopathic bacterial DNA in coronary stenotic artery plaque [corrected] and in dental plaque samples. J Clin Microbiol 2004;42:1313–5.
[34] Nakano K, Nemoto H, Nomura R, Inaba H, Yoshioka H, Taniguchi K, et al. Detection of oral bacteria in cardiovascular specimens. Oral Microbiol Immunol 2009;24:64–8.
[35] Azarpazhooh A, Leake JL. Systematic review of the association between respiratory diseases and oral health. J Periodontol 2006;77:1465–82.
[36] Leitich H, Bodner-Adler B, Brunbauer M, Kaider A, Egarter C, Husslein P. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. Am J Obstet Gynecol 2003;189:139–47.
[37] Pretorius C, Jagatt A, Lamont RF. The relationship between periodontal disease, bacterial vaginosis, and preterm birth. J Perinat Med 2007;35:93–9.
[38] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047–53.
[39] Janket SJ, Wightman A, Baird AE, Van Dyke TE, Jones JA. Does periodontal treatment improve glycemic control in diabetic patients? A meta-analysis of intervention studies J Dent Res 2005;84:1154–9.
[40] Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe 2011;10:497–506.
[41] Challacombe SJ, Shirilaw PJ. Immunity of diseases of the oral cavity. In: Ogra PL, Mestechky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. San Diego, CA: Academic Press; 1999. p. 1313–37.
[42] Gibbons RJ, Nygaard M. Interbacterial aggregation of plaque bacteria. Arch Oral Biol 1970;15:1397–400.
[43] Theilade E, Theilade J, Mikkelsen L. Microbiological studies on early dentogingival plaque on teeth and Mylar strips in humans. J Periodontal Res 1982;17:12–25.
[44] Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105:1135–43.
[45] Kurita-Ochiai T, Yamamoto M. Periodontal pathogens and atherosclerosis: implications of inflammation and oxidative modification of LDL. Biomed Res Int 2014:595981. http://dx.doi.org/10.1155/2014/595981.