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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

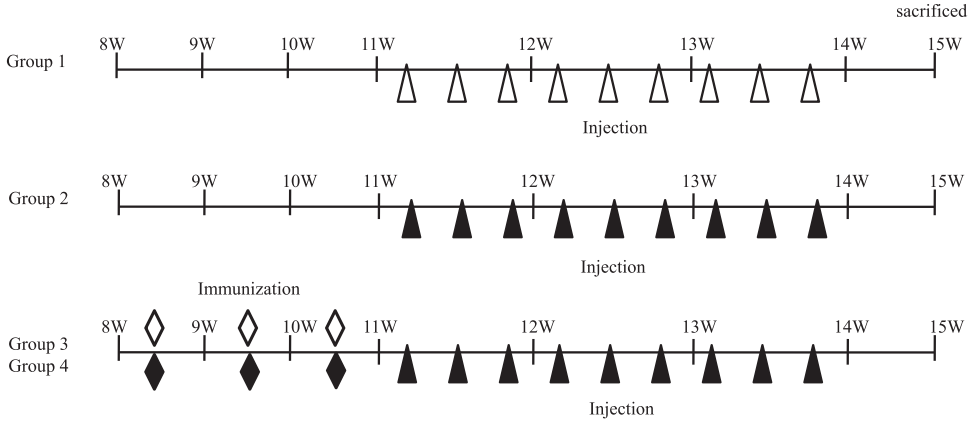

Fig. 1. Experimental procedure. Eight-week-old female Apoe^{shl} mice were randomly divided into 3 groups: Group 1 was inoculated with 100 μL of PBS (△), Group 2 was inoculated with 100 μL (10^8 CFU) of *P. gingivalis* (▲), Group 3 was immunized with 40k-OMP plus CpG ODN (○) and inoculated with 100 μL (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.