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Effects of egg yolk antibody against *Porphyromonas gingivalis* gingipains in periodontitis patients

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Abstract: ***Porphyromonas gingivalis*** **gingipains is suspected to be one of the most important causative agents of periodontitis. We postulated that the inhibition of gingipains may reduce the pathogenic nature of *P. gingivalis*. Anti-*P. gingivalis* egg yolk antibody (IgY-GP) was isolated from the yolks of hens immunized with purified gingipains. We applied IgY-GP gel subgingivally in periodontitis patients who harbored *P. gingivalis* in their subgingival flora. Five pairs of contralateral anterior single-rooted teeth were selected. One tooth in each contralateral pair was randomly treated with IgY-GP and subgingival scaling and root planing, whereas the other tooth was treated with SRP alone. The number of *P. gingivalis* bacteria was assessed by real-time PCR. Bacterial levels were expressed as the percentage of total bacteria. The IgY-GP group had a significant reduction in probing depth. BOP significantly decreased in the IgY-GP group compared to the control group at week 4. The levels of *P. gingivalis* significantly increased in the control group at week 4, whereas the reduction in the levels of *P. gingivalis* was sustained in the IgY-GP group. Within the limitations of the present study, IgY-GP was shown to be an effective immunotherapeutic agent in the treatment of periodontitis. (J. Oral Sci. 49, 201-206, 2007)**

Keywords: gingipains; IgY; periodontitis; *Porphyromonas gingivalis*.

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Introduction

Periodontitis is an inflammatory disorder that results in the destruction of the supporting structures of the teeth, including the alveolar bone. It is the most important cause of tooth loss among adults and is initiated by an accumulation of predominantly anaerobic Gram-negative bacteria in subgingival sites. Among the various bacterial species associated with the development of periodontitis, the Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is suspected to be one of the most important causative agents of the chronic form of this disease (1).

P. gingivalis produces a broad spectrum of virulence factors, lipopolysaccharide (2), fimbriae, hemagglutinin, hemolysin, and proteinase (3-6). Arg- and Lys-cysteine proteinases (gingipains) are the main endopeptidases produced by *P. gingivalis* (7). Gingipains play a major role in the progression of human periodontal disease, especially in host colonization, inactivation of host defenses, tissue destruction, and host immune system modulation (8-14). In addition, gingipains play a role in bacterial housekeeping (15), including amino acid uptake from host proteins, heme acquisition from erythrocytes (16), and fimbriae maturation (17). We postulated that the inhibition of gingipains may reduce the pathogenic nature of *P.*

gingivalis. Our previous investigation demonstrated that anti-*P. gingivalis* egg yolk antibody against gingipains (IgY-GP) inhibited gingipain activity *in vitro* (18). Consequently, we explored the possibility of *P. gingivalis* recolonization in humans and assessed its effect on periodontal disease. We applied IgY-GP gel subgingivally in periodontitis patients who harbored *P. gingivalis* in their subgingival flora.

Materials and Methods

Preparation of IgY-GP

Porphyromonas gingivalis (ATCC 33277) was maintained on Brucella HK agar (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% horse blood under anaerobic conditions. Log-phase *P. gingivalis* was harvested by centrifugation at 10,000 × *g* for 30 min at 4°C. After carefully removing the supernatant, the cell pellets were resuspended in previously cooled sterile Tris buffer (0.05 M Tris–1 M $CaCl_2$, pH 7.5) and then extracted by sonication (150 watts) for 6 turns (1 min/turn) in ice water, allowing 2 min between turns. Insoluble material from the sonicated cell suspension was separated by centrifugation at 10,000 × *g* for 15 min at 4°C, and the supernatant containing the crude gingipain antigen was collected. To purify the sonicates, the supernatant was passed through a Mono-Q fast-protein liquid chromatography column (Pharmacia, Uppsala, Sweden) pre-equilibrated with Tris-buffer at a flow rate of 60 ml/h. The gingipains were loaded onto the Mono-Q column and eluted with the same buffer containing 1 M NaCl. The major peak was observed during the early phase of NaCl elution and a minor peak was detected during the later phase. The major peak, which accounted for 80% of the total activity, was selected for further purification after adequate dialysis against Tris buffer (19). Purified gingipains were used in this study.

Five-month-old White Leghorn hens (strain HyLine W36; GHEN Corporation, Gifu, Japan) kept in conventional isolated poultry housing were immunized for egg antibody production. The hens were inoculated intramuscularly in the breast muscles with 1.0 ml of a vaccine (0.5 ml in each breast muscle) consisting of oil-adjuvant gingipain antigen (20). Eight weeks after the initial immunization, a booster immunization was administered in the same manner. Two weeks after the booster immunization, 60 eggs laid by the immunized hens were harvested and pooled when the antibody titer in the yolks peaked. Egg antibody powder was produced using a method similar to that described by Yokoyama et al. (20). Briefly, the yolks of the pooled eggs were separated from the albumin and yolk membrane. The egg yolk was homogenized with a mixer (HVM-106; Nihonseiki Kaisha, Tokyo, Japan) and filtered through Teflon filter cloth (Asamasu Co., Ltd., Nagoya, Japan). The filtrate was applied to a spray-dry machine (Model L-12; Ohkawara Kakohki, Kanagawa, Japan), which was operated at an air-inlet temperature of 140°C. The dried material was transported to the collection vat by a flow of air at 72°C. The dried antibody powder was stored in a desiccator at room temperature until use. Control IgY powder was prepared from the eggs of non-immunized hens in the same manner. Partially purified IgY-GP powder was prepared from egg yolk powder by chloroform (21) extraction and ammonium sulfate precipitation (22). IgY-GP was evaluated for specificity with an enzyme-linked immunosorbent assay (ELISA) (23).

Clinical evaluation

Five patients (mean age 59 years, range 50–67 years), who were referred to Nihon University School of Dentistry Dental Hospital (Tokyo, Japan) for periodontal treatment, were included in the study. All patients had at least 20 standing teeth and detectable levels of *P. gingivalis*, defined as at least 3% of the microbial flora in plaque samples evaluated by real-time polymerase chain reaction (PCR). All patients had received previous periodontal therapy involving oral hygiene instructions and had at least two probing pockets with a depth of ≥ 5 mm that bled on probing. Alveolar bone loss in each patient was determined with full mouth radiographs. All patients who participated in the study provided written informed consent. The study protocol was approved by the Nihon University School of Dentistry Institutional Review Board.

The study employed a split-mouth design. In total, five pairs of contralateral anterior single-rooted teeth were selected. Both teeth in each contralateral pair were required to exhibit gingival inflammation with bleeding on probing (BOP), subgingival calculus, and a probing depth > 4 mm in at least one aspect. One tooth in each contralateral pair was randomly treated with IgY-GP and subgingival scaling and root planing (SRP), whereas the other tooth was treated with SRP alone. The two treatment modalities were equally divided between the right and left sides of the mouth. All patients were treated by the same experienced practitioner. We assessed the clinical condition of the patients before treatment and 4 weeks post-treatment. After SRP, IgY-GP was administered directly into the periodontal pocket. Approximately 30–60 mg of 20% IgY-GP ointment (hydrocarbon gel, sucrose esters of fatty acids, hydroxypropylmethylcellulose 2208) was used to fill each pocket to the gingival margin.

We assessed the number of *P. gingivalis* bacteria in subgingival plaque samples collected from the treated teeth. The samples were collected using paper points

Table 1 Effects of IgY-GP on clinical parameters

	Probing Depth (mm)		Bleeding on Probing (%)	
	Baseline	4 weeks	Baseline	4 weeks
IgY-GP (N=5)	3.6 ± 0.6	2.7 ± 0.6 *	60.0 ± 19.0	10.0 ± 14.9 †
Control (N=5)	3.7 ± 0.4	3.1 ± 0.6	73.3 ± 25.3	56.7 ± 27.9

Data are expressed as mean ± SD. *$P < 0.05$ indicates a significant difference between values at baseline and week 4, according to Wilcoxon's signed-ranks test. †$P < 0.05$ indicates a significant difference between the IgY-GP group and control group at week 4, according to the Mann–Whitney *U*-test.

inserted into the periodontal pocket for 40 sec before treatment and 1, 2, and 4 weeks after treatment. The number of *P. gingivalis* bacteria was assessed by real-time PCR. Bacterial levels were expressed as the percentage of total bacteria (24).

Statistical analysis

The data were analyzed with SPSS software (SPSS, Chicago, IL). Differences in probing depth, BOP, and levels of *P. gingivalis* between the study groups were evaluated with the Mann–Whitney *U*-test. Differences between the groups at baseline and at week 4 were assessed with Wilcoxon's signed-ranks test. Significance was established at $P < 0.05$.

Results

The mean changes in probing depth and BOP from baseline to week 4 are shown in Table 1. The IgY-GP group showed a significant reduction in probing depth ($P < 0.05$). BOP was significantly decreased in the IgY-GP group compared to the control group at week 4. The levels of subgingival *P. gingivalis* for the SRP plus IgY-GP group and the SRP-alone group were determined with real-time PCR. Data are expressed as mean ± SD. Total bacteria number at baseline and at weeks 1, 2, and 4 was $1.94 \pm 3.63 \times 10^6$, $2.02 \pm 4.12 \times 10^6$, $5.68 \pm 12.60 \times 10^6$, and $1.30 \pm 1.36 \times 10^4$ in the control group, and $4.46 \pm 9.73 \times 10^6$, $2.75 \pm 3.12 \times 10^5$, $1.30 \pm 2.91 \times 10^7$, and $2.19 \pm 2.55 \times 10^4$ in the IgY-GP group, respectively. The levels of *P. gingivalis* significantly increased in the control group at week 4, whereas the reduction in the levels of *P. gingivalis* was sustained in the IgY-GP group (Fig. 1). No side effects were observed in the IgY-GP group.

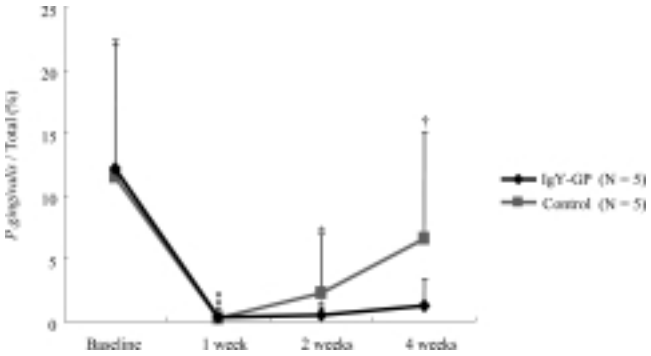

Fig. 1 Effects of IgY-GP administration on the levels of subgingival *P. gingivalis* in the periodontitis patients. The levels of subgingival *P. gingivalis* for the SRP plus IgY-GP group and the SRP-alone group were determined with real-time PCR. Data are expressed as mean ± SD. Samples were collected from the periodontal pocket before treatment and at weeks 1, 2, and 4 after treatment. Bacterial levels were expressed as the percentage of the total bacteria. *$P < 0.05$ indicates a significant difference compared to baseline, according to Wilcoxon's signed-ranks test. †$P < 0.05$ indicates a significant difference between values at weeks 1 and 4 week, according to Wilcoxon's signed-ranks test.

Discussion

The present study demonstrated that anti-*P. gingivalis* egg yolk antibody against gingipains can delay *P. gingivalis* recolonization in periodontitis patients. Passive immunization with antibodies from several different species has been used as immunotherapy against dental caries (25,26) and periodontitis (27-32). Previous studies have shown that antibodies are actively transported to the egg yolk of immunized hens (33). The use of IgY for passive immunization circumvents the need to use genetically modified organisms or to bleed animals to prepare antibodies. The production of polyclonal antibodies in eggs is convenient and economical because up to 40 mg of IgY can be obtained from a single egg (34). Successful passive immunization with IgY against dental caries has

been reported in a rat model (35,36) and in human subjects (2). However, due to the difficulties associated with developing effective animal models and the multifaceted nature of periodontitis, passive immunization therapy against periodontitis has not been studied extensively (37).

Gingipains degrade cytokines (10,11), components of the complement system (8,9), and several receptors, including macrophage CD14 (13,14,38) and T-cells CD4 and CD8 (39), thereby perturbing the host defense system and facilitating sustained colonization of *P. gingivalis*. Although it has been reported that gingival epithelial cells act as a physical barrier against the entry of periodontopathic bacteria, *P. gingivalis* can pass through the epithelial barrier (16,40). Previous reports have suggested that the proteolytic activities of gingipains affect epithelial cells by loosening the epithelial tissue from the basement membrane (41). These observations suggest that gingipains are the most promising target for vaccination against periodontitis and related systemic diseases. Immunization studies with gingipains have demonstrated protective effects against *P. gingivalis* infections in animal models (42,43).

In the present study, local administration of IgY-GP in periodontitis patients demonstrated a positive therapeutic effect at week 4. This long-term protection could not be explained by the persistence of IgY-GP in the periodontal pocket. Rather, it may have resulted from a shift in the microflora balance favoring the neutralization of *P. gingivalis* pathogenicity. This effect may be associated with the inhibitory effects of IgY-GP on the direct destruction of periodontal tissue and the disruption of normal host defense mechanisms induced by gingipains. Furthermore, IgY-GP may inhibit functions associated with the growth and survival of *P. gingivalis*, such as hemagglutination and the acquisition of heme and amino acids. This, in turn, may shift the ecological balance of the oral flora, inhibiting the recolonization of *P. gingivalis* in periodontal pockets after SRP. Following disruption of the bacterial biofilm by SRP and inhibition of *P. gingivalis* by IgY-GP administration, early colonizers, which are predominantly nonpathogenic, may be faster in occupying the vacant habitat, and thus result in reduced *P. gingivalis* levels (44,45).

In summary, IgY-GP suppressed the levels of *P. gingivalis* in the subgingival flora of periodontitis patients. Additional clinical studies using IgY as control may provide further evidence for the adjunctive use of IgY-GP ointment in periodontal therapy. Within the limitations of the present study, IgY-GP was shown to be an effective immunotherapeutic agent in the treatment of periodontitis.

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