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Short communication

Activity of anti-*Porphyromonas gingivalis* egg yolk antibody against gingipains *in vitro*

K. Yokoyama[1], N. Sugano[2,3], A. K. M. S. Rahman[4], M. Oshikawa[2], K. Ito[2,3]

[1]Nihon University Graduate School of Dentistry, Tokyo, Japan, [2]Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan, [3]Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan, [4]Immunology Research Institute, GHEN Corporation, Gifu, Japan

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Introduction: We investigated the effect of anti-*Porphyromonas gingivalis* egg yolk antibody against gingipains [immunoglobulin Y (IgY)-GP] on gingipain activity *in vitro*.
Methods: IgY-GP was isolated from the yolks of White Leghorn hens immunized with purified gingipains. Control antibody (IgY) was isolated from the yolks of non-immunized hens. Gingipain activity was assessed according to the rate of enzymatic substrate hydrolysis. Human epithelial cells were cultured with or without gingipains and with gingipains pretreated with either IgY-GP or IgY.
Results: Hydrolytic activity decreased in the presence of IgY-GP. Cells incubated with gingipains showed a dose-dependent loss of adhesion activity. Pretreatment of gingipains with IgY-GP was associated with strong inhibition of cell detachment, whereas pretreatment with IgY was not.
Conclusion: Our findings suggest that IgY-GP may be an effective immunotherapeutic agent in the treatment of periodontitis.

Key words: gingipains; immunoglobulin Y; *Porphyromonas gingivalis*

Naoyuki Sugano, Department of Periodontology, Nihon University School of Dentistry, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Tel.: +81 3 3219 8107;
fax: +81 3 3219 8349;
e-mail: sugano-n@dent.nihon-u.ac.jp
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Porphyromonas gingivalis, a gram-negative anaerobe, has been identified as an important periodontal pathogen because of its virulence (9, 22, 23, 37). Several virulence factors have been associated with the pathogenicity of *P. gingivalis*, including lipopolysaccharide (16), fimbriae, hemagglutinin, hemolysin (12, 14, 18, 21), and the Arg-X-specific (Rgp) and Lys-X-specific (Kgp) cysteine proteinases (the gingipains) (30). 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 (3, 10, 26, 29, 37, 38, 42). Surface proteinases, which include gingipains, have also been reported to be major virulence factors of *P. gingivalis* (31). Gingipains play a role in bacterial housekeeping (6), including amino acid uptake from host proteins, in heme acquisition from erythrocytes (34) and in maturation of fimbriae (28). We postulated that the inhibition of gingipains mighty reduce the pathogenesis of *P. gingivalis* so we investigated the effect of anti-*P. gingivalis* egg yolk antibody against gingipains [immunoglobulin Y (IgY) -GP] on enzymatic activity *in vitro*.

Material and methods

Preparation of IgY-GP

P. gingivalis (ATCC 33277) was maintained on Brucella HK agar (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% horse blood under anaerobic conditions (7). 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–HCl, 1 M $CaCl_2$, pH 7.5) and then extracted by sonication (150 watts) for six cycles (1 min/cycle) in ice water, allowing 2 min between cycles. 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 a later phase. The major peak, which accounted

for 80% of the total activity, was collected and used as partially purified gingipains for further study after adequate dialysis against Tris buffer (47).

Five 5-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 (1 mg/ml; 0.5 ml in each breast muscle) of a vaccine consisting of gingipain antigen with oil as adjuvant (43). Eight weeks after the initial immunization, a booster immunization was administered in the same manner. Sixty eggs were harvested and pooled. These eggs laid from two weeks after the booster onwards when an antibody titer in the yolk is peak. Egg antibody powder was produced using a method similar to that described by Yokoyama et al. (43). 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 a 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 and IgY powder were prepared from egg yolk powder by chloroform extraction (39) and ammonium sulfate precipitation (20). Activity of IgY-GP was evaluated using an enzyme-linked immunosorbent assay (44). Microdilution plates (Immulon 2; Dynatech Laboratories, Alexandria, VA) were coated with a 5-μg/ml solution of gingipains in 0.05 M carbonate buffer (pH 9.6; 100 μl/well) at 4°C for 18 h. The plates were emptied and blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (150 μl/well) at 37°C for 1 h and then washed with 0.02% Tween-20–PBS three times. IgY-GP and Ig-Y were reconstituted or suspended in PBS (8.4 mg/ml). Antibody solution in 0.05% Tween-20–PBS (100 μl/well) was incubated at 37°C for 1 h and the plates were washed as described above. Rabbit anti-chicken IgG conjugated with horseradish peroxidase (dilution of 1 : 8000; Cappel; Organon Teknika Co., Westchester, PA) in 0.05% Tween-20–PBS was applied and incubated at 25°C for 30 min; *o*-phenylenediamine and dihydrochloride were then added. The color reaction was stopped after 20 min with 1.5 M sulfuric acid, and the color intensity was measured at 490 nm.

Inhibition of enzymatic activity by IgY-GP

N-α-Benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA) was obtained from Sigma Chemical Co. (St Louis, MO). Gingipains (62.5, 125 and 250 μg/ml) were activated in a buffer consisting of 200 mM HEPES, 5 mM $CaCl_2$ (pH 7.6), and 10 mM cysteine for 5 min at 37°C, mixed with IgY-GP or IgY (50 mg/ml) in the same buffer, and then incubated at 4°C for 1 h.

A 50-μl aliquot of the pretreated sample was added to 150 μl of the reaction mixture, which consisted of 100 mM Tris–HCl buffer (pH 7.5) containing 5 mM dithiothreitol, 5 mM L-cysteine, and 2.5 mM BA*p*NA. The mixture was incubated at 37°C for 25 min, and the reaction was stopped by adding 50 μl 20% acetic acid. The release of *p*-nitroaniline was determined by measuring its absorbance at 405 nm (45). The substrate without enzyme served as the negative control and was used to monitor background readings. One unit of gingipain activity was defined as the amount of enzyme releasing 1 μmol *p*-nitroanilide per minute in the reaction mixture under assay conditions, and was expressed as U/ml.

Human oral epithelial cells (Ca9-22) were cultured overnight in Eagle's minimum essential medium containing 10% fetal bovine serum and antibiotics in six-well microtiter plates. The plates were charged with serum-free medium in the absence and presence of gingipains (62.5, 125, and 250 μg/ml) and with gingipains pretreated with either IgY-GP or IgY (50 mg/ml each) and incubated at 37°C for 1 h. The attached cells were counted after Trypan blue staining.

Statistical analysis

The data were analyzed with SPSS software (SPSS, Chicago, IL). Enzymatic activity was analyzed using Student's *t*-test. Significance was established at $P < 0.01$.

Results and discussion

The immune response of IgY-GP was assessed using an enzyme-linked immunosorbent assay. Table 1 shows that IgY-GP was active in immunization in the antigen-coated wells but was not active in the non-coated wells. IgY from non-immunized hens did not show activity in the antigen-coated wells.

Table 1. Characterization of IgY-GP and IgY

Types of IgY	OD value	
	Coated well	Non-coated well
PBS–Tween	0.025	0.044
IgY	0.026	0.030
IgY-GP	1.264	0.042

Data shown are the means of three independent experiments.

IgY-GP exhibited significant inhibitory activity against gingipains compared to the non-immunized egg yolk controls (Fig. 1). In the presence of IgY-GP, the activity of solutions containing 62.5, 125 and 250 μg/ml gingipains decreased by 60.5 ± 0.3, 59.6 ± 0.31 and 47.1 ± 1.8%, respectively.

Gingipains have been shown to disrupt human cell adhesion and to contribute to the tissue damage in periodontal disease caused by *P. gingivalis*. Purified gingipains, with or without pretreatment with IgY-GP, were added to cultures of Ca9-22 cells to investigate the effect of IgY-GP treatment on the gingipain-induced detachment of epithelial cells. The cells incubated with gingipains showed a dose-dependent loss of adhesion activity. Pretreatment of gingipains with IgY-GP strongly inhibited the gingipain-induced detachment of cells

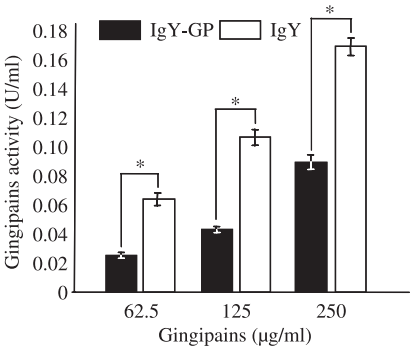

Fig. 1. Effects of IgY-GP on the protease activity of gingipains. The inhibitory effects of IgY-GP and IgY (50 mg/ml each) on gingipain activity were evaluated using the synthetic substrate *N*-α-benzoyl-L-arginine-*p*-nitroanilide. The release of *p*-nitroaniline was determined by measuring its absorbance at 405 nm. One unit of gingipain activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitroanilide per minute in the reaction mixture under assay conditions, and was expressed as U/ml. Data are shown as the means ± SD of three independent experiments. $*P < 0.01$ indicates a significant difference between study groups according to Student's *t*-test.

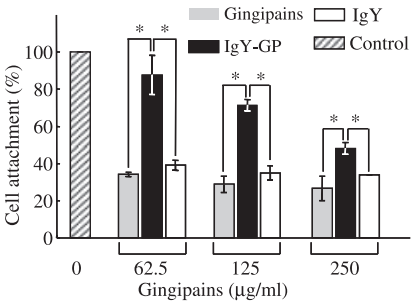

Fig. 2. Effects of IgY-GP on the detachment of human epithelial cells induced by gingipains. Human epithelial cells were incubated in serum-free Eagle's minimal essential medium with or without gingipains and with gingipains pretreated with either IgY-GP or IgY (50 mg/ml each) for 1 h. The attached cells were counted after Trypan blue staining. Data are shown as the means ± SD of three independent experiments. $*P < 0.01$ indicates a significant difference between the study groups according to Student's *t*-test.

(Fig. 2), whereas pretreatment with IgY did not.

The present study demonstrated that anti-*P. gingivalis* egg yolk antibody against gingipains can inhibit gingipain activity *in vitro*. Passive immunization with antibodies from several different species has been used as immunotherapy against dental caries (24, 40) and periodontitis (4, 5, 8, 27, 41, 46), and previous studies have shown that antibodies are actively transported to the egg yolk of immunized hens (25). 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 IgY may be obtained from a single egg (32). Successful passive immunization with IgY against dental caries has been reported in a rat model (15, 36) and in human subjects (17). However, because of the difficulties associated with developing effective animal models and the multifaceted nature of periodontitis, passive immunization therapy against periodontitis has not been studied as extensively (1).

Gingipains degrade cytokines (3, 26), components of the complement system (10, 42), and several receptors, including macrophage CD14 (11, 37, 38) and T-cell CD4 and CD8 (19), thereby perturbing the host defense system and facilitating sustained colonization of *P. gingivalis*. Although it has been suggested that gingival epithelial cells act as a physical barrier against the entry of periodontopathic bacteria, *P. gingivalis* can pass through the epithelial barrier (33, 34). Previous reports have suggested that the proteolytic activities of gingipains affect epithelial cells by loosening the epithelial tissue from the basement membrane (2). 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 (13, 31).

In the present study, IgY-GP strongly inhibited gingipain activity and the gingipain-induced detachment of cells *in vitro*. 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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