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Antibody response of periodontal patients to Porphyromonas gingivalis heat shock protein

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I. Introduction

Periodontal disease is composed of complex disease entities with etiological components comprising complex microorganisms,[1,2] with their complex antigenic components. Diverse immunological mechanisms and immune recognition of antigen in periodontal diseases[3] render the immune responses not necessarily beneficial to host inherent from the cross-reactivity, ecological commensalisms or antagonisms[4,5]. These bacteria may include, but not limited to, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans, Prevotella intermedia, Treponema denticola, Bacteroides forsythus, Campylobacter rectus* and *Fusobacterium nucleatum*[1].

HSP 60 has been found to be a common antigen of many bacterial pathogens[6] and its cross-reactivity were reported[7]. HSP genes have been cloned in *P. gingivalis*[8], *A. actinomycetemcomitans*[9], *B. forsythus*[10], *T. denticola*[11], *F. nucleatum*[12], *C. rectus*[13], and *P. intermedia*[14]. HSPs have also been suggested to be strongly associated with autoimmune destructive mechanism in periodontal disease[15-17] and atherosclerosis[18] due to bacterial HSP sequence shares a moderate level of sequence homology with mammalian counterpart.

We have selected HSP as a candidate antigen for investigating antibody response due to the following important reasons; 1) HSP can be found in all of the selected principal periodontopathogenic organisms, 2) HSP-mediated autoimmune mechanism is being characterized both in periodontal disease and atherosclerosis, thus study of HSP may lead to valuable information, 3) GroEL or DnaK genes has been extensively cloned in periodontopathogenic bacteria. Among HSPs from several periodontopathogenic bacteria, we have selected *P. gingivalis* HSP due to the following reasons; 1) *P. gingivalis* has been the most important key pathogen in destructive periodontal diseases[2], 2) we have been concentrating on establishment of *P. gingivalis* HSP-specific T cell lines in periodontal and atherosclerosis patients, 3) HSP from such as *F. nucleatum* is more likely to be

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associated with autoimmune mechanism[19]. Therefore, it may be critical to pick up *P. gingivalis* HSP as a strong candidate antigen for which humoral responses develop in the immunopathogensis of periodontal diseases. The present study has been performed to evaluate antibody response to *P. gingivalis* heat shock protein in periodontal patients.

II. Materials and Methods

1. Patient selection

Patients who were diagnosed and suffering from adult periodontitis have been reviewed and examined. Informed consent forms were obtained from them after being informed of periodontal therapy. Subjects without history of periodontal disease were defined as the control group I.

2. Purification of recombinant P. gingivalis heat shock protein 60

P. gingivalis GroEL gene was introduced into a glutathoine S-transferase-*P. gingivalis* GroEL fusion construct in pGEX-4T-3 expression vector (*P. gingivalis* GroEL gene was cloned (8) and kindly provided by Professor Yoji Murayama, Okayama University Dental School, Japan). The fusion construct was transformed into HB-1142 cells and used to inoculate a 250ml overnight culture grown in L-broth containing 100 mg/ml ampicillin at 37℃ at 180rpm. Protein expression was induced by the addition of 1M isopropyl b-D-thiogalactoside (IPTG) to a final concentration of 1mM followed by incubation at 30 ℃ at 270rpm for 180min. Cells were harvested at 6,000 rpm 10 min. Cell pellets from 250 ml of induced culture were resuspended in 25ml of buffer A (100 mM TEA-HCl, 170 mM NaCl, 1 % Triton X-100, 10 mM dithiothreitol, pH 7.4). The resuspended pellets were sonicated on ice in a sonicator using a medium probe at 14-16 mm amplitude. The sonicate was centrifuged at 13,000 rpm for 10 min. The supernatants were transferred to fresh tubes. To each tube approximately 2 ml of a 1:2 slurry of glutathione Sepharose 4B beads (Pharmacia, Sweden), washed twice buffer B (PBS+1 % Triton X-100), was added and the tubes were incubated at room temperature on a rotating wheel for 60 min to allow the fusion protein to bind to the beads. The beads were pelleted by centrifugation at 12,000 rpm for 5 min, resuspended in 10ml of buffer B and washed three gently in 10 ml of buffer B. The fusion protein was eluted from the beads with 10 x 1 ml fractions of elution buffer (10 mM reduced Glutathione in 50 mM Tris-HCl, pH 8.0) at room temperature for 10min.

3. Measurement of serum IgG antibody titers by ELISA

Sera were obtained from peripheral blood from the patients and determined of anti-*P. gingivalis* hsp60 IgG by a slight modification of the method previously reported[20-25]. Microtiter plates were coated in triplicate with 100 ml of hsp (10 mg/ml) diluted in phosphate buffer. After overnight incubation at 4°C, the plates were washed three times with PBS containing 0.05 % Tween 20 (PBS/Tween). A total of 50 ml of serum samples serially diluted in PBS/Tween was added to each well and incubated for 2 hours at room temperature. The plates were washed three times with PBS/Tween, and 100 ml of peroxidase-conjugated mouse anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were added. After being incubated for 2 hours at room temperature, the plates were washed three

times with PBS/Tween. Aliquot of 100 ml of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and incubated for 15 min at room temperature followed by adding 100 ml of 0.18 M H_2SO_4 to stop the reaction. Optical densities read at 450 nm of wavelength were plotted as a function of serum dilution factor, and regression analysis was performed. One of the control group I sera (periodontally healthy subjects without history of cardiovascular diseases) was assigned an ELISA unit of 100 and serum IgG titer of other control group I subjects and the atherosclerosis patients were calculated. Antibody titer was assigned to be elevated if it is higher than the control titer + 3 x standard deviation.

4. Establishment and characterization of P. gingivalis heat shock protein-specific T cell lines

P. gingivalis-specific T cell lines from peripheral blood were established according to the method previously described[20-23]. Peripheral blood mononuclear cells were also isolated by gradient cell separation technique using Ficoll-Paque medium (Pharmacia, Upsala, Sweden). Using 12-well tissue culture plates (Costar, Corning, Corning, NY), mononuclear cells (1 x 10^6 cells/well) isolated from peripheral blood were stimulated with *P. gingivalis* (1 x 10^8 cells/ml) together with antigen presenting cells (APC, 5 x 10^6 cells/well) after treatment with mitomycin C (25 mg/ml). Peripheral blood lymphocytes were additionally drawn from the patients, T cells were removed by nylon wool, and non-T cell fraction was used as APCs. After 2 weeks of incubation, T cells were allowed to rest for 1 week. After the resting period, fresh mitomycin-treated non-T cells and *P. gingivalis* were added again to induce T cell proliferation.

From *P. gingivalis*-specific T cell lines, *P. gingivalis* hsp-specific T cell lines were established in the same manner by adding the heat shock protein antigen (5 mg/well in 12-well culture plate) in alternating cycles for stimulating T cell lines. Culture supernatants were harvested and preserved at 20°C until used for cytokine assay.

For characterization of T cell lines, cells were double-stained with Per-CP-conjugated mouse anti-human CD3, FITC-conjugated mouse anti-human CD4 or PE-conjugated mouse anti-human CD8 monoclonal antibodies (PharMingen, San Diego, CA). Phenotypic expression of each T cell line was screened by flow cytometry using an Epics Elite ESP (Coulter, Hialeah, FL).

5. Determination of cytokine concentrations

To determine IFN-gamma, IL-4, or IL-10 concentration of the culture supernatants, a sandwich ELISA was employed according to the method described previously[20-23]. Briefly, 96-well plates (Corning, Coming, NY) were coated with mouse anti-human IFN-gamma, IL-4 or IL-10 (PharMingen, San Diego, CA, 4 mg/*ml*) diluted in sodium carbonate buffer overnight at 4°C. After washing three times with PBS/Tween, wells were blocked by PBS + 10 % fetal bovine serum (PBS/FBS) for 30 min at room temperature and then washed three times with PBS/Tween. Each sample and standard recombinant human IFN-gamma, IL-4 or IL-10 (PharMingen, San Diego, CA, 4 ng/*ml*) or PBS diluted in PBS/FBS + 0.05% Tween20 (PBS/FBS/Tween), as the positive or negative control, respectively, were added, and incubated for 3 hours at room temperature and the plates were washed three times with PBS/Tween. Biotinylated mouse anti-human IFN-gamma, IL-4 or IL-10 (PharMingen, San Diego, CA, 2 mg/*ml* in

PBS/FBS/Tween) were added to each well and incubated for 1 hour at room temperature. After washing four times with PBS/Tween, hydroperoxidase-conjugated streptavidin (PharMingen, San Diego, CA, 2 mg/m1 in PBS/FBS/Tween) were added and incubated for 30 minutes at 37°C. The plates were washed 8 times with PBS/Tween and o-phenylenediamine (1 mg/ml in 0.1 M citrate buffer, pH 4.5) was added and incubated for 20 min at room temperature. To stop the color reaction, 4 N H_2SO_4 was added to each well and the optical densities were read at wavelength of 490 nm. Optical densities of standard cytokines were plotted against the dilution factors and cytokine concentration of each sample was determined.

III. Results

1. Patient selection

50 patients (aged between 43-62, Mean 46.9) who were diagnosed and suffering from adult periodontitis have been selected while 50 subjects (aged between 22-28, mean 24.3) without history of periodontal disease were defined as the control group.

2. Purification of recombinant P. gingivalis heat shock protein 60

Purified recombinant *P. gingivalis* HSP 60 was subject to SDS-PAGE for confirmation of size and purity (Figure 1).

3. Measurement of serum IgG antibody titers by ELISA

Anti-*P. gingivalis* hsp60 IgG antibody titers of 50 periodontitis patients ranged from 216.5 to 290.8, all of which being considered to be elevated than

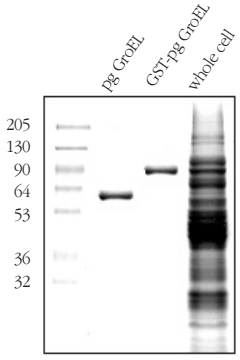

Figure 1. SDS-PAGE picture of purified recombinant *P. gingivalis* GroEl 60 (Lane 1).

Table 1. Mean anti-*P. gingivalis* heat shock protein IgG antibody titer of sera from periodontitis patients and control subjects[a]

Patients	Control group
(N=50)	(N=50)
246.6±18.9[b]	102.9±10.5
(216.5-290.8)	(101.2-103.7)

[a] result from triplicate measurements(mean±s.d.)

[b] titer higher than controls±3 s.d

Table 2. Mean cytokine concentration of culture supernatants of *P. gingivalis*-specific T cell lines from peripheral blood (ng/ml)a

IFN-gamma	IL-4	IL-10
4.5±1.7 (3.1-5.8)	2.6 ±1.1 (2.2-3.2)	1.7±0.6 (1.6-1.9)

the control group (Table 1). Mean anti-*P. gingivalis* hsp IgG antibody titer of 50 control subjects were 102.9.

4. Establishment and characterization of P. gingivalis heat shock protein-specific T cell lines

CD3+/CD4+ or CD3+/CD8+ T cells of *P. gingivalis* hsp-specific T cell lines from peripheral blood of periodontitis patients demonstrated varying ranges of phenotypic profiles (45.1-66.6 % for CD4+ and 19.8-44.3 % for CD8+ T cells), respectively (data not shown).

5. Determination of cytokine concentrations

Culture supernatants of *P. gingivalis* hsp-specific T cell lines produced variable amounts of IFN-gamma (3.1-5.8 ng/ml), IL-4 (2.2-3.2 ng/ml) and IL-10 (1.6-1.9 ng/ml) (Table 2).

IV. Discussion

For multiple targeting of these pathogenic bacteria, knowledge of cross-reactive antigen(s) which share(s) a high degree of sequence homology is essential. Heat shock proteins (HSP) and phosphorylcholine (PC) could fit into candidate antigens. HSP facilitates protein folding and translocation across membrane barriers possibly by secretion. HSP 60 has been found to be a common antigen of many bacterial pathogens[6] and its cross-reactivity were reported[7]. HSP genes have been cloned in *P. gingivalis*[8], *A. actinomycetemcomitans*[9], *B. forsythus*[10], *T. denticola*[11], *F. nucleatum*[12], *C. rectus*[13], and *P. intermedia*[14]. HSPs have also been suggested to be strongly associated with autoimmune destructive mechanism in periodontal disease[15-17] and atherosclerosis[18] due to bacterial HSP sequence shares a moderate level of sequence homology with mammalian counterpart. PC can also be found commonly in oral bacteria and demonstrates a considerable cross-reactivity among oral bacteria[26]. Its association with periodontal disease has also been reported[27].

Among these two bacterial antigens (i.e. HSP and PC) 1) which share significant sequence homology among oral bacteria and 2) of which immunopathogenic mechanisms have been postulated in periodontal diseases, we have selected HSP as a candidate antigen for vaccine development for the following important reasons; 1) HSP can be found in all of the selected principal periodontopathogenic organisms, while PC was not reported in *P. gingivalis*, 2) HSP-mediated autoimmune mechanism is being characterized both in periodontal disease and atherosclerosis, thus study of HSP may lead to valuable information, 3) GroEL or DnaK genes has been extensively cloned in periodontopathogenic bacteria.

In the present study, we found the elevated antibody responses to *P. gingivalis* HSP 60 in periodontitis which were significantly higher than those of control healthy subjects. This indicates the possible involvement of *P. gingivalis* HSP 60 in the autoimmune destruction of periodontal tissues targeting gingival fibroblasts and endothelial cells. It should

be interesting how this autoimmune destructive mechanism may operate in periodontal destructive process. *P. gingivalis* HSP 60-specific T cell response could also be demonstrated in the present study, indicating the involvement of HSP 60-mediated cellular immune mechanism in the pathogenesis of periodontal disease. Each T cell line produced variable amounts of IFN-gamma, IL-4 and IL-10 indicating mixture of TH1 and TH2 cells. Flow cytometric analysis also indicated the polarization of CD8+ cells which might have also contributed to the production of these cytokines. Yet, this is a preliminary observation that *P. gingivalis* HSP 60 may be involved in the pathogensis of periodontal diseases as well as in atherosclerosis[20].

V. References

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-국문초록-

P. gingivalis 열충격단백에 대한 치주질환자의 항체반응

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재조합 *P. gingivalis* 열충격단백에 대한 치주질환자의 항체반응과 세포성 면역반을을 검사한 결과, 항체역가는 건강군의 역가에 비해 통계적으로 유의성 있게 상승되어 있었고, 항원특이성 T 세포면역반응을 관찰할 수 있었다. 이러한 결과로 미루어보아 *P. gingivalis* 열충격단백은 치주질환의 면역병리기전에 관여한다는 것을 관찰할 수 있었다.

주요어 : heat shock protein, antibody, Porphyromonas gingivalis, periodontitis (열충격단백, 항체, Porphyromonas gingivalis, 치주염)