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Epitope mapping of heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis, a putative pathogen in human periodontal disease, possesses a 60-kDa heat shock protein (hsp60, GroEL). The GroEL homologs are known to be key molecules in auto-immune reactions because of the sequence similarity with human hsp60. In this study, B-cell epitopes on *P. gingivalis* GroEL (PgGroEL) were analyzed by both Western immunoblotting with truncated PgGroEL and by the multi-pin synthetic peptide approach. To examine auto-antibody production in periodontitis patients, Western immunoblotting with human gingival fibroblasts was performed. Deletion mutants were constructed from the cloned PgGroEL gene (*P. gingivalis groEL*), and four C-terminal truncated PgGroEL and one N-terminal truncated PgGroEL were prepared from the deletants. Sera from periodontitis patients reacted with all truncated PgGroEL used in this study. The results suggest that the B-cell epitopes were overlaid throughout PgGroEL. To determine the detailed locations of the B-cell epitope, 84 decapeptides covering the entire PgGroEL were synthesized and the serum IgG response to the peptides was examined. Epitope mapping using the synthetic peptides confirmed that the B-cell epitopes were overlaid throughout the length of PgGroEL and revealed that highly conserved peptides between PgGroEL and human hsp60 were recognized by the serum antibodies. Immuno-reactivity against human gingival fibroblasts was examined with sera from 30 periodontitis patients and 10 periodontally healthy subjects. IgG antibody against the 65-kDa antigen in human gingival fibroblasts (same molecular mass as human hsp60) was detected in two patients. Although IgG production against human hsp60 may be rare case in periodontitis patients, the results of epitope mapping demonstrated the potential of PgGroEL to cause the cross-reactions with human hsp60.

Keywords: Heat shock protein 60 (GroEL); B-cell epitope; *Porphyromonas gingivalis*

1. Introduction

Heat shock proteins (hsp) are highly conserved through evolution and are structurally similar in all living organisms, so that immune responses against hsp can cross-react and produce anti-self reactivity [1]. Immune responses to the cross-reactive determinants in bacterial hsp are thought to be generated early in life and the immunologic memory may function as a 'common barrier' against infections [2]. On the other hand, cross-reactive determinants in human hsp may function as a trigger for auto-immune reactions [3,4].

Periodontal disease is a chronic infectious disease, with *Porphyromonas gingivalis* being one of the most frequently implicated pathogens [5–10]. Compared with other pathogens, periodontal bacteria persist for many years or decades in periodontal pockets and thus present a long-term challenge, including bacterial hsp, to the host immune system. We hypothesize that continuous exposure to hsp from periodontal bacteria may cause cross-reactions with human hsp and that the cross-reactions may modify destructive forms of periodontal tissue.

Among hsp families, hsp60 (GroEL) homologs are major heat shock protein antigens in various bacterial infections [11]. They are antigenically cross-reactive and serologically detectable in a wide range of Gram-negative bacteria as to be considered key molecules for auto-immune reactions [1–4,12–15]. Our previous study revealed the complete nucleotide sequence of the gene for *P. gingivalis* GroEL (PgGroEL) and reported the purification

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method of its recombinant protein (r-GroEL) [16]. PgGroEL showed sequence similarity with human hsp60 and r-GroEL was a highly antigenic protein which was frequently recognized by sera from patients with periodontitis [16]. PgGroEL may have cross-reactive determinants with human hsp60. Therefore, mapping of B-cell epitopes on PgGroEL is important to investigate the potential of PgGroEL as a trigger for auto-immune reactions. In addition, it is crucial to examine the profiles of auto-antibody production in periodontitis patients for a better understanding of auto-immunity in the pathogenicity of periodontal disease. In the current study, we examined the reactivity of IgG antibodies in sera from periodontitis patients with truncated PgGroEL and 84 synthetic decapeptides covering the entire PgGroEL to identify continuous B-cell epitopes. Furthermore, Western immunoblotting was performed with human gingival fibroblasts to detect auto-antibodies in patients with periodontitis.

2. Materials and methods

2.1. Construction of deletion mutants

Deletion mutants of *P. gingivalis groEL* were constructed as previously described [16]. Briefly, genomic DNA of *P. gingivalis* 381 was digested with *Hin*dIII and then a 2.6-kbp DNA fragment containing *P. gingivalis groEL* was inserted to the *Hin*dIII site of pUC18. The inserted DNA was deleted with exonuclease III from the *Xba*I site (from the C-terminal end of PgGroEL) of the vector. The reaction for the deletion was stopped every minute (1 to 5 min) to obtain various lengths of the insert. Then the deleted plasmids were blunt-ended by Mung bean nuclease and were self-ligated. The ligation mixture was transformed to *Escherichia coli* XL1-blue and then the protein profiles of the transformants were analyzed by SDS–PAGE for isolation of clones which produce truncated PgGroEL. The plasmids in the isolated clones were recovered and the nucleotide sequences of the inserted DNA were analyzed using a *Taq* dye deoxy terminator cycle sequencing kit and a 373 A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Antigen preparation

The transformant *E. coli* which expressed the truncated PgGroEL was disrupted by sonication and the sonic extracts were then prepared as described previously [17]. The extracts were separated by SDS–PAGE and the gel regions corresponding to the truncated PgGroEL were excised. Each truncated PgGroEL in the excised gel was eluted and recovered from the gel using an Electro Eluter model 422 (Bio-Rad, Hercules, CA, USA), and was subjected to Western immunoblotting. Human gingival fibroblasts were isolated from five patients with periodontitis and were cultured as described previously [18]. A sonic extract of the cultured gingival fibroblasts was prepared by the same method employed for the transformant *E. coli* and was subjected to Western immunoblotting.

2.3. Human sera

Thirty patients' sera were selected from the serum sample collection of Okayama University Dental Hospital. Serum antibody titers to the sonic extract (whole cell) of *P. gingivalis* [17] and the serum reactivity to the r-GroEL (complete length) had previously been examined for the serum selection [16]. The 30 patients' sera selected in this study had elevated IgG antibody titers (above 400 ELISA units [17]) to the sonic extract of *P. gingivalis* and had IgG antibodies to r-GroEL. Ten periodontally healthy subjects were also used as controls (less than 40 ELISA units).

2.4. Western immunoblotting

Western immunoblotting was performed as described previously [19]. Sera from patients with periodontitis and healthy subjects were used at a final dilution of 1:500 with 5% (wt/vol) skimmed milk in Tris-buffered saline (10 mM Tris–HCl buffer (pH 7.5), 0.9% NaCl; M-TBS). Anti-*Yersinia enterocolitica* hsp60 monoclonal antibody (Wako Pure Chemical Industries, Osaka, Japan) was used at a dilution of 1:1000 in the M-TBS. Horseradish peroxidase-conjugated goat anti-human IgG antibody (1:1000 dilution with M-TBS; INC Biochemicals, Costa Mesa, CA, USA) or horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:1000 dilution with M-TBS; Organon Teknika Corp., West Chester, PA, USA) were used for detection.

2.5. Epitope mapping

A total of 84 decapeptides covering the entire amino acid sequence of PgGroEL were synthesized using an Epitope-Scanning Kit (Chiron Mimotopes, Clayton, Victoria, Australia) according to the manufacturer's instructions based on Fmoc chemistry. Peptides were designed to overlap by five amino acid residues only in the highly conserved regions or in the regions specific to *P. gingivalis*. The success of the synthesis was monitored by simultaneous synthesis of a positive (PLAQ) and a negative (GLAQ) control peptide and by subsequently testing their binding to the supplied monoclonal antibody. The synthesized peptides, coupled to the surface of the polypropylene pins configured to a 96-well microtiter plate, were tested for binding with sera using a modified ELISA. The pins were pre-coated for 1 h in a microtiter plate containing 2% bovine serum albumin (BSA) and 0.1% Tween 20 in 10 mM PBS (pH 7.2) (PBS-T). After the pre-coating, the pins were incubated overnight at 4°C in sera (1:1000 dilution with pre-coated buffer). They were then washed four times

with PBS-T and incubated for 1 h with alkaline phosphatase-conjugated goat anti-human IgG (1:5000 dilution with 0.1% sodium caseinate in PBS-T; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). After four washes with PBS-T, the pins were placed in the wells of a microtiter plate containing a *p*-nitrophenylphosphate substrate solution [17] and color development was allowed to proceed for approximately 30 min at 37°C. The reaction was stopped by removing the pins from the substrate solution, and the plates were then read at 405 nm in a microtiter plate reader (model 550: Bio-Rad). After testing with one serum sample, antibodies bound to the peptides were removed by the method previously described [20] and the pins were ready for testing with the next serum sample.

3. Results and discussion

3.1. Reactivity of sera to truncated PgGroEL

Four deletion mutants producing C-terminal truncated PgGroEL were isolated by the recombinant DNA procedure. The protein profiles of the deletants are shown in Fig. 1A. Plasmids in the deletants were isolated and the nucleotide sequences of the insert DNA were analyzed. Each deletant contained a 1378-bp, 1265-bp, 1189-bp and 844-bp open reading frame (ORF) of the *groEL* gene (the complete *P. gingivalis groEL* consists of 1635 bp) and expressed 50-kDa, 46-kDa, 44-kDa and 34-kDa proteins, respectively. Another recombinant clone producing both the complete length of PgGroEL (65 kDa) and the N-terminal truncated PgGroEL (58 kDa) has already been isolated [16] and was subsequently used in this study. The length of each truncated PgGroEL is shown in Fig. 1C. These truncated PgGroEL expressed in *E. coli* were purified and subjected to Western immunoblotting with the patients' sera. Ten sera were chosen at random from the 30 sera selected in this study and used for the Western immunoblotting. A representative result is shown in Fig. 1B. All sera reacted with all truncated PgGroEL. The results suggest that the B-cell epitopes overlay throughout PgGroEL or that the epitopes may exist only at the middle region of PgGroEL. We employed the multi-pin peptide technique for further characterization of the B-cell epitopes.

3.2. Reactivity of sera to human gingival fibroblasts

The reactivity of sera to the sonic extract of human gingival fibroblasts was analyzed using sera from 30 periodontitis patients and 10 healthy subjects (Fig. 2). Anti-*Y. enterocolitica* hsp60 monoclonal antibody, which is known to cross-react with human hsp60 [21], reacted with a 65-kDa human gingival fibroblast antigen. Although very few

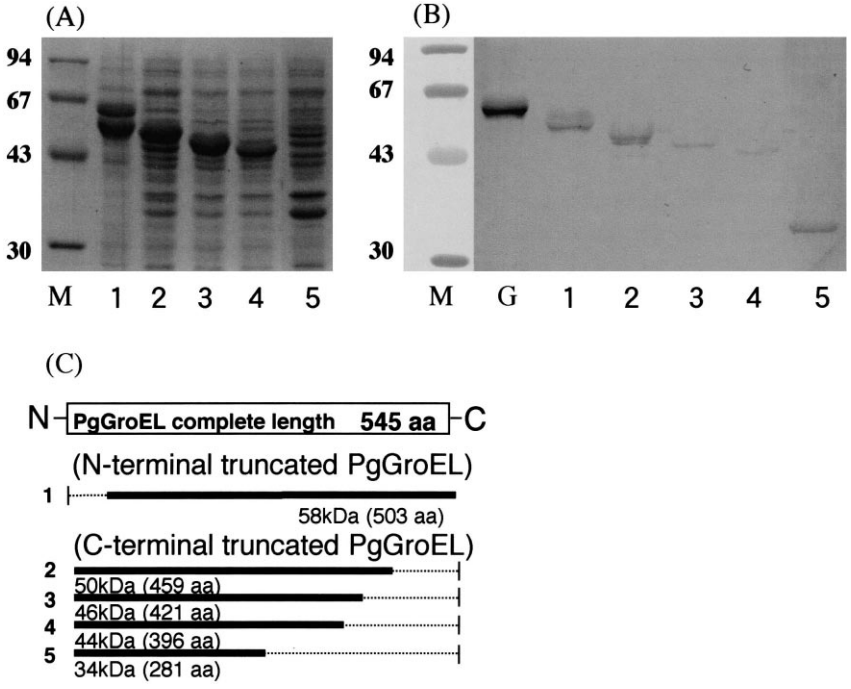

Fig. 1. SDS–PAGE and Western blot profiles of truncated PgGroEL. A: Coomassie brilliant blue-stained SDS–PAGE gel of the deletion mutants expressing both the complete length of GroEL and N-terminal truncated PgGroEL (lane 1) and the C-terminal truncated PgGroEL (lanes 2–5). B: Western blot analysis of patient's serum to truncated PgGroEL. The complete length of PgGroEL (lane G), N-terminal truncated PgGroEL (lane 1) and C-terminal truncated PgGroEL (lanes 2–5) were probed with sera from periodontitis patients. Ten sera were picked up at random from the 30 sera selected in this study and were absorbed with excess *E. coli* sonic extracts (without plasmid) before use to reduce backgrounds. Lane M shows molecular mass standards, and the numbers to the left of the gels are molecular masses (in thousands). C: Alignment figure of truncated PgGroEL. The numbers on the left side of the panel correspond with the lane numbers in the above figures.

subjects reacted with any fibroblast antigens, three out of the 30 patient's sera did react to the antigens and two of these three showed reactivity to an antigen at the same molecular mass (65 kDa) as the antigen detected by anti-*Y. enterocolitica* hsp60 monoclonal antibody. The three sera also reacted with another fibroblast antigen at a different molecular mass from human hsp60. No remarkable difference was observed among the five cell lines.

As for auto-antibody production in periodontal disease, several groups have reported elevations of anti-collagen antibodies in diseased sites or in patients' serum [22–25]. The results of Western immunoblotting suggest that some periodontitis patients may develop auto-antibodies against gingival fibroblasts in serum except for collagen and that the sera may include IgG antibody against human hsp60.

3.3. Reactivity of sera to synthetic peptides

Nine patient's sera, including three sera reacting with fibroblast antigens, and one healthy subject were used for epitope mapping by multi-pin peptide approach. Fig. 3 indicates representative epitope-scanning patterns. Epitope-scanning patterns were relatively similar among the patient's sera tested in this study except one serum. A specific pattern was found in one serum (serum no. 2 in Figs. 2 and 3) which showed reactivity to the 65-kDa human gingival fibroblast antigen. Interestingly, this serum showed a striking ELISA signal to a highly conserved peptide between PgGroEL (MQFDRGYISP; shown by * in Fig. 3) and human hsp60 (MKFDRGYISP). The serum from the healthy subject gave weak signals with all peptides.

The means and the standard deviations of the ELISA signals to the peptides were calculated in each serum sample. A datum line (mean+1 standard deviation) was determined and each signal to the peptide was evaluated as positive or negative according to the line. Eighteen antigenic peptides, which showed positive signals in more than five patients, were determined to be major B-cell epitopes. The epitopes identified are shown in Fig. 4 with other hsp60 homologs on which B-cell epitopes have already been reported [20,26]. Eighteen regions were identified as major B-cell epitopes throughout the length of PgGroEL.

Yi et al. have reported B-cell epitopes on the *Chlamydia*

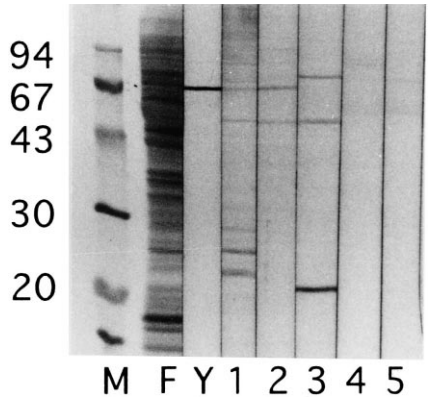

Fig. 2. Serum response to fibroblast antigens. Lane M, molecular mass standard marker; lane F, sonic extract of human gingival fibroblasts stained with amido black; lane Y, human hsp60 detected by anti-*Y. enterocolitica* hsp60 monoclonal antibody; lanes 1–5, responses of sera from patients. Each lane contains 15 μg of protein. The numbers on the left are molecular masses (in thousands). No detectable band was observed in most subjects (partially shown in lanes 4 and 5). Two of the 30 patients' sera reacted with the 65-kDa fibroblast antigen (lanes 1 and 2). No remarkable difference was observed among cell lines.

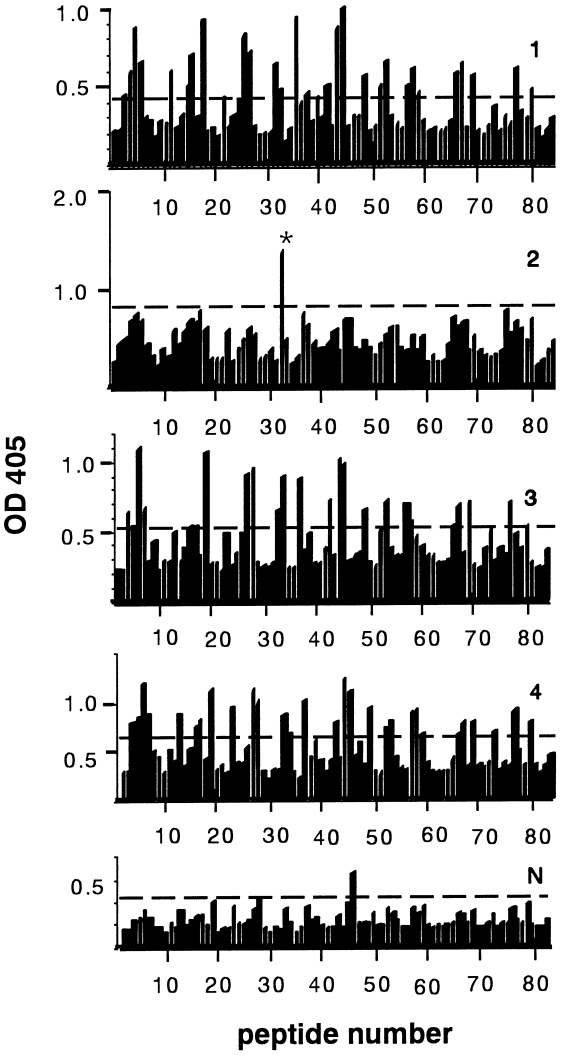

Fig. 3. Serum response to synthetic peptides. The reactivity of sera from patients and a healthy subject to the 84 decapeptides covering the entire PgGroEL are shown by optical density. The peptides were numbered starting from the N-terminal region. Strong ELISA signals indicate highly antigenic peptides on PgGroEL. Highly antigenic peptides were most common among the patients' sera (nos. 1, 3 and 4 are representative results) except one subject (no. 2). The serum showing a specific epitope-scanning pattern was the one which reacted with the 65-kDa fibroblast antigen (lane 2 in Fig. 2). This serum showed a strong ELISA signal to the peptide shown with an asterisk. The amino acid sequence of this peptide was highly conserved between PgGroEL (MQFDRGYISP) and human hsp60 (MKFDRGYISP). The mean+1 S.D. of the optical density in each scan is shown with a broken line. The subject numbers in this figure correspond to the lane numbers in Fig. 2. Subject N is a healthy control.

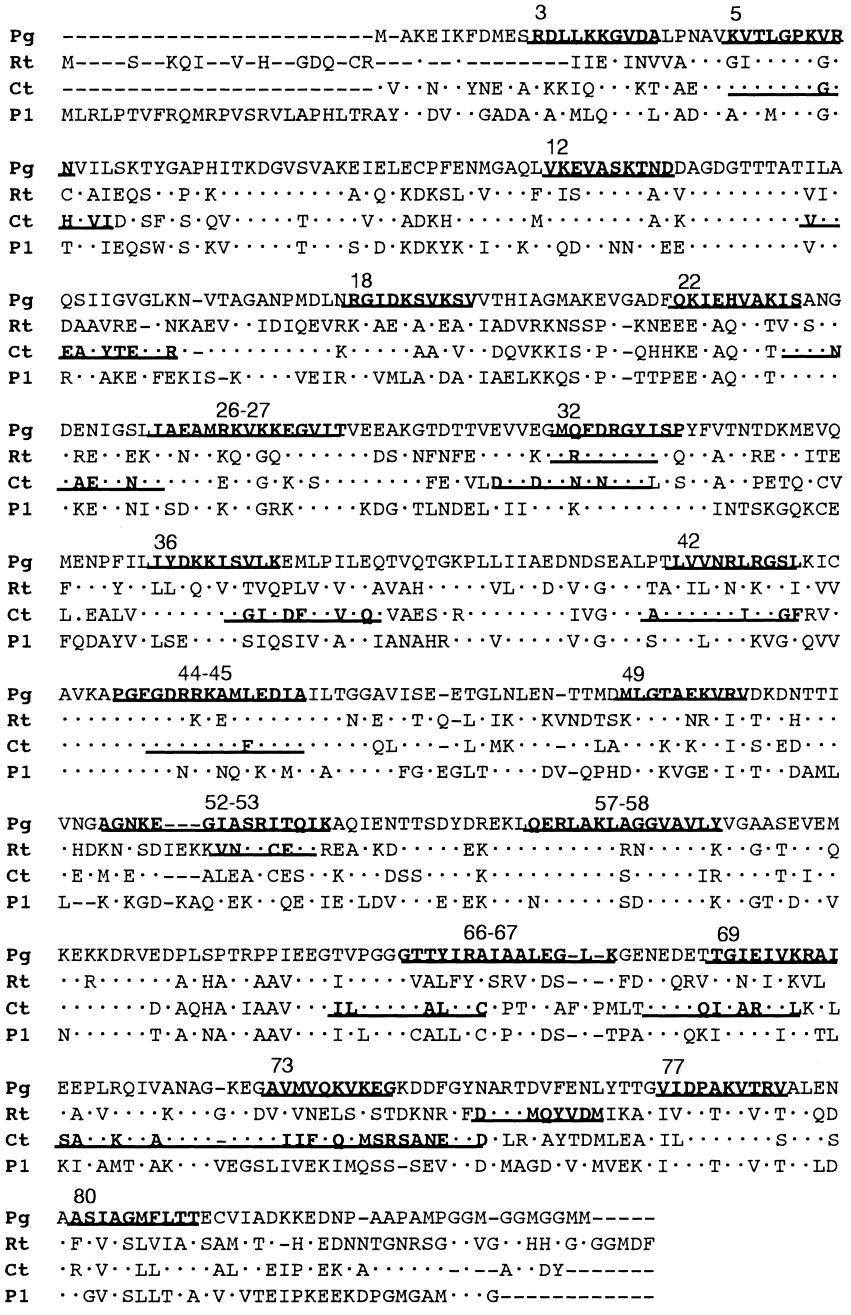

Fig. 4. Major B-cell epitopes on PgGroEL. Major B-cell epitopes on PgGroEL are shown with epitopes of other hsp60 homologs. The amino acid sequence of PgGroEL is shown with the corresponding published sequences of *R. tsutsugamushi* Sta58 protein (Rt), *C. trachomatis* hsp60 (Ct) and human P1 protein (P1). Dot marks in the figure indicate the same amino acid sequences of other hsp60 homologs with PgGroEL and short bars indicate spaces for the alignment. B-cell epitopes on PgGroEL are shown underlined with the numbers of the synthetic peptides. Published B-cell epitopes of other hsp60 homologs are also underlined [20,26].

trachomatis hsp60 and cross-reactive epitopes on human hsp60 [26]. We identified B-cell epitopes in the same regions (peptides 5, 32, 42, 44–45, 52–53, 69 and 73 in Fig. 4) as *C. trachomatis* hsp60. The epitope regions of PgGroEL seem to correspond well with those of *C. trachomatis* hsp60. Highly antigenic regions might be common among hsp60 homologs. Highly antigenic and conserved peptides could be key determinants for autoimmune reactions. Peptide numbers 5, 32, 42, 44–45, 57–58, and 69 showed more than 60% identity with human hsp60. In particular, the peptide number 32 (MQFDRGYISP), showing a striking ELISA signal with serum no. 2, has a 90% similarity with human hsp60. The region corresponding to peptide number 32 has been identified in another bacterial GroEL as a major B-cell epitope. Octapeptide (MRFDRGYI) in the Sta58 major outer protein of *Rickettsia tsutsugamushi* was frequently recognized with sera from scrub typhus patients [20]. This region might be

a key determinant for the cross-reaction between GroEL homologs and human hsp60. However, contrary to these results, the sera from our patients generally did not react with human hsp60 in human gingival fibroblasts for unknown reasons. The conformational epitopes may be major targets of the immune responses to PgGroEL. Petit et al. have reported the protective role of immune responses to hsp in periodontitis patients using mycobacterial hsp as antigens [27]. In the same way, the reactivity to PgGroEL may be protective in periodontitis patients in most cases.

Auto-immune reactions might be rare cases in periodontal patients. However since some patients with periodontitis (2/30) developed IgG antibodies against human gingival fibroblasts including the 65-kDa antigen, auto-immune reactions may occur in periodontal lesion and may be a critical risk factor for some patients. Further elucidation is needed to evaluate the influence of auto-antibodies on the clinical aspects. Although there are many points still unknown, including the mechanism and process of anti-self antibody induction, results of this study could suggest the potential of PgGroEL to cause cross-reactions with human hsp60.

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