T- and cross-reactive B-cell epitopes of *Porphyromonas gingivalis* and human heat shock protein 60 in atherosclerosis

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I. INTRODUCTION

Periodontal infection may be one of the risk factors for cardiovascular diseases.[1, 2, 3] This association is supported by the recent observation that *Porphyromonas gingivalis* (*P. gingivalis*), a primary periodontopathic pathogen, can invade the endothelial cells.[4, 5] This pathogen can not only elaborate proinflammatory cytokine of the endothelium that recruits the inflammatory cells into the atherosclerotic lesions, but also modulate production of interleukin-8 and monocyte chemotactic protein 1 in human vascular endothelial cells.[6-8]

Due to considerably high degree of sequence homology between bacterial and human heat shock proteins (hsp), this protein might be involved in autoimmune disease mechanisms operating in humans.[9, 10] T cell immune responses specific to bacterial or human hsp have been demonstrated in atherosclerosis.[11-13] Host immune system primed by hsp of a major periodontal pathogen, such as *Porphyromonas gingivalis* (*P. gingivalis*), can cross-react with cognate mammalian counterpart in gingival connective tissue or arterial walls.[14, 15] To provide evidence that *P. gingivalis* may be actively involved in immunopathogenic process of atherosclerosis, we have recently reported T cell response specific to *P. gingivalis* or *P. gingivalis* hsp in atherosclerosis patients.[2, 16]

It is critical to identify immunodominant epitope(s) of an infecting pathogen that is (are) exclusively recognized by T- and/or B-cells in clarifying the immune mechanisms modulating the autoimmune diseases. In the present study, we have attempted to identify T- and/or B-cell epitopes of *P. gingivalis* hsp60, and cross-reactive B-cell epitopes of human hsp60 in atherosclerosis. We have also evaluated the HLA restriction patterns of T-cells in atherosclerosis patients.

II. MATERIALS AND METHODS

Patient selection criteria. Patients with atherosclerosis were screened for systemic and periodontal disease. Periodontal examination was done for measurement of probing pocket depths and level of

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attachment for each patient and subsequently classified according to the 6th edition of Current Procedural Terminology published by American Academy of Periodontology (American Academy of Periodontology, 1991). Patients who demonstrated destructive periodontal disease with the elevated serum IgG antibody responses to *P. gingivalis* were included in the study. Informed consent forms were obtained from patients. Clinical investigation has been conducted according to Declaration of Helsinki principles. Clinically healthy subjects without any noticeable history of systemic disease or periodontal disease were included as the control group.

Measurement of anti-*P. gingivalis*, anti-*P. gingivalis* hsp60 and anti-human hsp60 IgG antibody titers by ELISA. Microtiter plates coated with either formalinized *P. gingivalis* cells, *P. gingivalis* hsp60, or human hsp60, respectively, diluted in 10 mM phosphate buffer were incubated with an aliquot of serum samples.[2,16] After washing, peroxidase-conjugated mouse anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were added. The plates were washed and an aliquot of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for incubation followed by adding 0.18 M H_2SO_4 to stop the reaction. Optical densities were plotted as a function of the serum dilution factor for determination of the titer. Antibody titer was considered to be elevated if it was higher than mean control titer + 3 x standard deviation.

Synthetic peptide. A total of 108 decapeptides spanning the entire amino acid sequence of *P. gingivalis* GroEL were synthesized using an Epitope-Scanning Kit (Chiron Mimotopes, Clayton, Victoria, Australia). A total of 108 decapeptides of human hsp60 corresponding to each peptide sequence were also synthesized in the same protocol. Peptides were designed to overlap by five amino acid residues.

T cell epitope mapping. T cells (1×10^5 cells/well) from *P. gingivalis* hsp60-reactive T cell lines[16] were stimulated with synthetic peptides (5 mg/ml) and APC (5×10^6 cells/well). After 48 hours of incubation, the cells were labeled with ^{3}H-thymidine for incubation of additional 6 hours and counted in a liquid scintillation counter. Proliferation was presented as the stimulation index (SI); the ratio of the mean counts per minute (cpm) with antigen to the cpm without antigen. SI values of 2 or greater were considered to be positive.

Preparation of conjugated plate. Conjugation of synthetic peptide to the microtiter plate (CovaLink plate, NUNC, Denmark) was done with a water-soluble 1-ethyl-(3-dimethyl-aminopropyl) (EDC) carbodiimide in the presence of N-hydroxy-succinamide (NHS). Each peptide was dissolved in dimethyl sulfoxide and diluted with 0.1M carbonate-bicarbonate buffer and used for coating plates.

B-cell epitope mapping. The conjugated plate was washed and aliquots of serum samples was added and incubated. After washing the plates, peroxidase-conjugated mouse anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were added. The plates were incubated, washed and an aliquot of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for incubation followed by adding 0.18 M H_2SO_4. Optical density means and the standard deviations of the ELISA signals to the peptides were calculated for each sample. The mean + 1 standard deviation was set up as a bottom line and each signal to the peptide was assigned as positive or negative responses.

Dot immunoblot. Recombinant *P. gingivalis* hsp60,[16] human hsp60 (StressGen Biotechnology, Victoria, Canada), respectively, were spotted on the

nylon membrane. After blocking the membrane, human sera were added for incubation. The membrane was washed followed by adding horseradish peroxidase-conjugated mouse anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing the membrane, tetramethylbenzidine was added for color development.

HLA-DRB and -DQB genotyping. HLA-DRB1 and DQB1 genotyping of T-cells was done by PCR-SSOP (polymerase chain reaction-based sequence-specific oligonucleotide probe) hybridization using HLA-DRB and DQB typing kit (Dynal RELI™ SSO system, DYNAL Biotech, Wirral, U.K.). Briefly, genomic DNA was isolated from T-cells from each T-cell line by the phenol-chloroform extraction method. The polymorphic region in the second exons of the HLA-DRB, -DQB genes were PCR-amplified with group-specific primers in the typing kit by an automated programmable thermocycler. The PCR mixture consisted of 200 to 300 ng of genomic DNA, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0 at 25℃), 0.1 % Triton ×-100, 200 mM dNTPs, 1 mM each primer, and 2.5 units of the *Taq* DNA polymerase. The mixture (50 or 100 ml) was subjected to 30 cycles of denaturation at 96°C for 1 minute, annealing at an appropriate temperature for 1 minute, and extension at 72℃ for 2 minutes. The PCR-amplified products were hybridized with a panel of 45 or 25 biotinylated SSOP in the strips, respectively, for the identification of subtypes in the DRB1 or DQB1 loci.

III. RESULTS

Patients. Six atherosclerosis patients, diagnosed as having arteriosclerosis obliterans in right or left superficial femoral arteries, or both, were screened and subject to surgical intervention of atherosclerotic plaques. All of them were males, aged between 57-73, and smokers. They demonstrated the severe periodontitis.[16]

Anti-*P. gingivalis*, anti-*P. gingivalis* hsp60, and anti-human hsp60 IgG antibody titers

Mean anti-*P. gingivalis*, anti-*P. gingivalis* hsp60, or anti-human hsp60 IgG antibody titers of all six atherosclerosis patients were elevated when compared with those of ten control subjects (Table 1).

T-cell epitope mapping. T-cells from each patient showed multiple reactivity to twenty peptides of which the SI value were 2 or greater. Of the twenty peptides, ten peptides were designated as the major T-cell epitopes that were identified as T-cell epitopes in more than four out of six patients. These were peptide nos. 3, 15, 24, 33, 45, 53, 64, 84, 88, 99 (Table 2).

B-cell epitope mapping. Twenty antigenic peptides that showed positive signals in more than four out of six patients, were designated as major B-cell epitopes of *P. gingivalis* hsp60. The epitopes as represented in Figure 1 were peptide nos. 3, 6, 15, 24, 29, 33, 39, 45, 53, 56, 64, 69, 74, 75, 84, 85, 88, 93, 99, 102. In the same manner, major B-cell epitopes of human hsp60 were identified. These included peptide nos. 15, 29, 53, 56, 69, 74 (Figure 1, Table

Table 1. Serum IgG antibody titers against *P. gingivalis*, *P. gingivalis* hsp60 or human hsp60 in atherosclerosis patients.

	Antigens		
Mean titers	*P. gingivalis*	*P. gingivalis* hsp60	human hsp60
Patients (N=6)	1,256 + 61.5	186.1 + 21.6	211.8 + 16.9
Control (N=10)	106.4 + 12.1	102.6 + 8.1	108.2 + 11.3

Table 2. List of amino acid sequences of ten peptides of *P. gingivalis* hsp60 that were identified as major T-cell epitopes.

peptide no.	position	amino acid sequence
3	12-21	RDLLKKGVDA
15	73-82	VKEVASKTND
24	117-126	RGIDKSVKSV
33	162-171	IAEAMRKVKK
45	222-231	IYDKKISVLK
53	262-271	LVVNRLRGSL
64	316-325	MLGTAEKVRV
84	416-425	GTTYIRAIAA
88	438-447	TGIEIVKRAI
99	493-502	VIDPAKVTRV

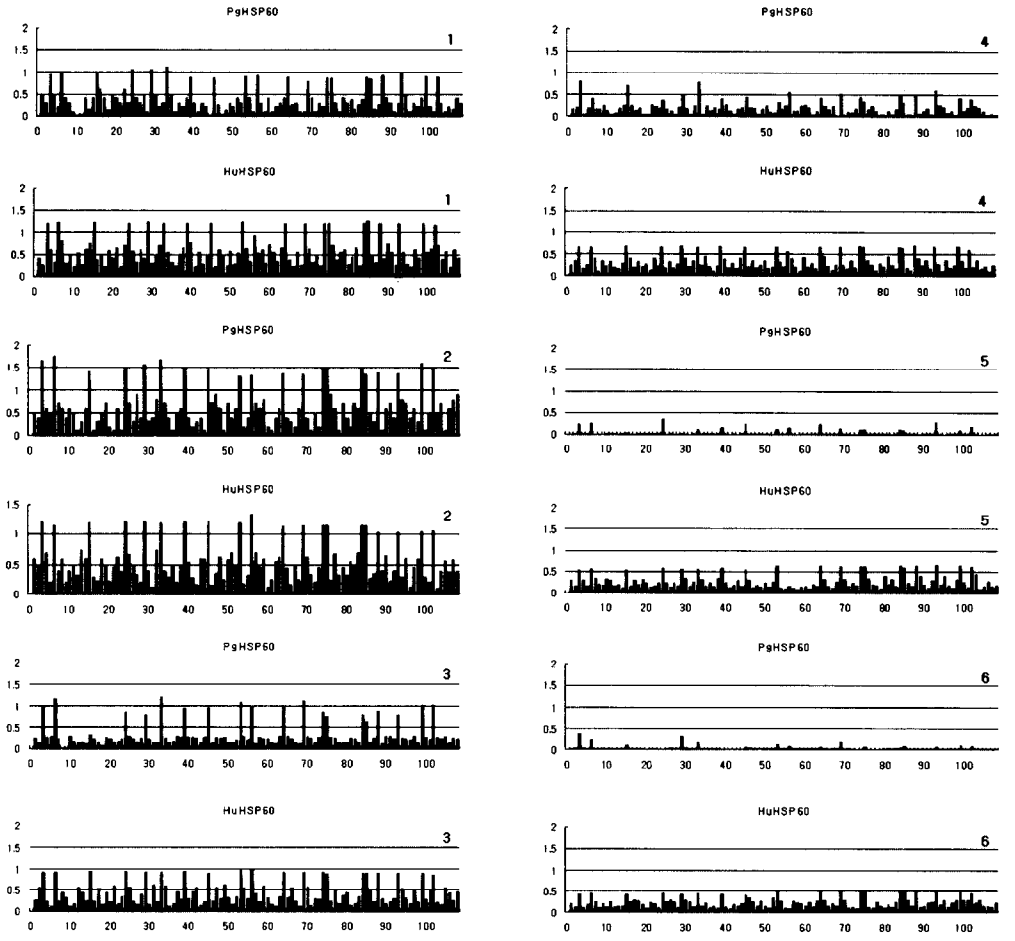

Figure 1. Bar diagrammatic representation of optical densities as a measure of serum reactivity to 108 synthetic peptides spanning whole *P. gingivalis* hsp60 and human hsp60, respectively. The number indicates each patient.

Table 3. Cross-reactive B-cell epitopes for *P. gingivalis* hsp60 and human hsp60.

Patient #	*P. gingival* HSP60	human HSP60**
1	20 peptides*	6,**15**,**29**,**53**,**56**,85
2	20 peptides	3,6,**15**,24,**29**,33,39,45,**53**,**56**,**69**,**74**,75,84,85
3	20 peptides	3,6,**15**,24,**29**,33,39,**53**,**56**,64,**69**
4	20 peptides	**15**,**29**,33,39,**56**,**74**,88,93,99
5	20 peptides	**53**,**56**,64,**69**,**74**,75,84,88,93,99,102
6	20 peptides	**53**,**56**,64,**69**,**74**,75,84,85,88,93

* 20 peptides include the peptide numbers 3, 6, 15, 24, 29, 33, 39, 45, 53, 56, 64, 69, 74, 75, 84, 85, 88, 93, 99, 102 of P. gingivalis hsp60
** bold numbers indicate peptides numbers of human hsp60 that were identified as B-cell epitopes in more than four out of six patients

Table 4. Amino acid sequences of common B-cell epitopes of *P. gingivalis* hsp60 and human hsp60, respectively

Peptide no.	*P. gingival* Hsp60	human Hsp60
15	VKEVASKTND (aa 73-82)	VQDVANNTNE (aa 71-80)
29	QKIEHVAKIS (aa 142-151)	EEIAQVATIS (aa 140-149)
53	LVVNRLRGSL (aa 262-271)	LVLNRLKVGL (aa 260-269)
56	PGFGDRRKAM (aa 279-288)	PGFGDNRKNQ (aa 277-286
69	GIASRITQIK (aa 342-351)	QIEKRIQEII (aa 340-349)
74	QERLAKLAGG (aa 367-376)	NERLAKLSDG (aa 365-374)

Figure 2. Dot immunoblot pattern of cross-recognition of *P. gingivalis* hsp60 (P) and human hsp60 (H) by sera obtained from six atherosclerosis patients (nos. 1-6).

3). Hence, these peptides of human hsp60 with corresponding peptides of *P. gingivalis* hsp60 were designated as cross-reactive B-cell epitopes of *P. gingivalis* hsp60 and human hsp60, respectively. The amino acid sequences of these peptides were compared in Table 4.

Dot Immunoblot. Sera from atherosclerosis patients who had elevated IgG antibody to *P. gingi-*

Table 5. HLA-DRB1 and DQB1 alleles in each patient and T-cell epitope of *P. gingivalis* hsp60

Patient #	HLA -DRB1	HLA -DQB1	*P. gingival* hsp60 T-cell epitopes
1	1504	0603	20 peptides except 29, 53, 64, 74
2	1504	0203	20 peptides except 3, 6, 29, 45, 74, 85
3	1504	0603	20 peptides except 6, 69, 85
4	1504	0603	20 peptides except 56, 69, 93, 99
5	0413	0603	20 peptides except 39, 56, 75, 93, 102
6	1504	0603	20 peptides except 24, 39, 56, 102

* 20 peptides include the peptide numbers 3, 6, 15, 24, 29, 33, 39, 45, 53, 56, 64, 69, 74, 75, 84, 85, 88, 93, 99, 102 of *P. gingivalis* hsp60

valis hsp60 demonstrated strong cross-reactive patterns with *P. gingivalis* hsp60 and human hsp60 as evidenced by dot immunoblot. Sera from healthy control reacted neither with *P. gingivalis* hsp60 nor with human hsp60 (Figure 2).

HLA-DR and -DQ genotying

HLA-DR and -DQ types of T-cells from each patient are summarized in Table 5. Five out of six patients were positive for HLA-DRB1*1504 allele except for one patient whose genotype was positive for DRB1*0413. Five out of six patients were positive for HLA-DQB1*0603 type with one patient positive for DQB1*0203 was found in another patient.

IV. DISCUSSION

Mean anti-*P. gingivalis*, anti-*P. gingivalis* hsp60 or human hsp60 IgG antibody titers in six atherosclerosis patients in the experimental group were higher when compared with the control subjects. However, there is a controversial opinion regarding the reactivity of sera to human hsp60 in atherosclerosis.[17, 18] Sera from atherosclerosis patients who had elevated IgG antibody to *P. gingivalis* hsp60 demonstrated a cross-reactivity with human hsp60 antibody titers as evidenced by dot immunoblot, suggesting the autoimmune pathogenic mechanisms regulated by *P. gingivalis* hsp in human atherosclerosis.[9, 10, 14-16] Recently, several authors claimed the critical role of bacterial stress proteins or human hsp in recruiting immune cells which target antigens, consequently leading to the development of plaque and atheroma lesions.[11, 12]

Hsp are highly conserved and in case of hsp60, approximately 60 % sequence homology between the mycobacterial and human cognates has been observed.[19] Hence, T cells and antibodies with specificity for conserved sequences are potentially auto-reactive contributing to pathogenesis of infectious diseases, including periodontal disease.[14, 15, 20] Previously, we could establish *P. gingivalis* hsp-reactive T cell lines from mononuclear cells isolated from atheromatous plaque in all six patients.[16] The T-cells were a mixture of $CD4^+$ and $CD8^+$ cells producing the cytokines characteristic of both Th1 and Th2 subsets, suggesting *P. gingivalis* hsp-specific T cells home to the atheroma lesion where *P. gingivalis* have infiltrated.[4,5]

P. gingivalis GroEL gene was originally cloned and sequences by Maeda et al.[21] In the present study, we have performed to scan anti-*P. gingivalis* hsp60 and anti-human hsp60 serum antibody for specific linear B-cell epitopes on these proteins. Differences in antibody binding were observed in majority of patients. Interestingly, ten of these epitopes of *P. gingivalis* hsp60 identified as common T-cell and B-cell epi-

topes in atherosclerosis patients in the present study were identical to B-cell epitopes in periodontitis reported by others.[22] Interestingly, peptide nos.15, 29, 53, 56, 69 and 74 of *P. gingivalis* hsp60 and human hsp60, respectively, were identified as cross-reactive B-cell epitopes. Of these, peptide no. 15 of *P. gingivalis* hsp60 has also been identified as one of B-cell epitopes of periodontitis[22] and corresponding peptide no. 15 of human hsp60 has been identified as one of B-cell epitopes of atherosclerosis,[23] and T-cell epitopes in atherosclerosis.[24] Peptide no. 56 of *P. gingivalis* hsp60 and human hsp60, respectively, has consistently been identified as cross-reactive B-cell epitope in all the patients. Peptide no. 56 has also been identified as one of B-cell epitopes in periodontitis.[22]

The HLA systems control immune responses by presenting antigenic epitopes to immune T-cells. HLA molecules restrict and regulate the range of immune responses to different antigens and mediate susceptibility of resistance to infecting micro-organisms. The linkage between disease susceptibility and HLA molecules is ambiguous, however, can vary due to the distribution of HLA antigens in different populations.[25] Five out of six patients were positive for HLA-DRB1*1504 and HLA-DQB1*0603 indicating *P. gingivalis* hsp60 is processed and presented in HLA class II-restricted pattern. Mustafa et al.[26] have demonstrated the association of *Mycobacterium leprae* hsp65-reactive T-cell lines with HLA-DR53 encoded by HLA-DRB4 gene, while others have reported the association of *Chlamydial* hsp60 with HLA-DQB1*0102 and 0602 alleles.[25] These differences seem to be related to the different hsp antigens used in the studies. Epitope-specific association with multiple MHC class II molecules remains to be defined to establish the vaccine design in an HLA heterogeneous human population.[27]

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-국문초록-

동맥경화증에 있어서 *Porphyromonas gingivalis*와 인체 열충격단백의 T-세포 및 교차성 B-세포 epitope

최점일

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본 연구의 목적은 인간의 동맥경화증에서 *Porphyromonas gingivalis* (*P. gingivalis*)와 인체 열충격단백 60의 T-세포 및 교차성 B-세포 epitope를 규명하고 수립된 T-세포주의 T-세포 주요조직적합체 양상을 파악하려는 데 있다. *P. gingivalis* 열충격단백-반응성 T 세포주와 환자의 혈청을 이용하여 *P. gingivalis* 열충격단백60 분자를 구성하는 104개의 중복성 합성 펩타이드의 T-세포 epitope과 B-세포 epitope을 규명하였다. 인체 열충격단백60에 대한 B-세포 epitope도 같은 방법으로 파악하였다. *P. gingivalis*, *P. gingivalis* 열충격단백60 또는 인체 열충격단백60에 대한 IgG 항체는 모든 동맥경화증 환자에서 상승하였다. *P. gingivalis* 열충격단백60의 3, 15, 24, 33, 45, 53, 64, 84, 88, 99번 펩타이드가 주요한 T-세포 epitope였고 이것들은 T-세포 및 B-세포 공동 epitope이기도 했다. 또한 인체 열충격단백60 교차반응 B-세포 epitope은 15, 29, 53, 56, 69, 74번 펩타이드로 판명되었다. 대부분 환자의 주요조직적합체는 HLA-DRB1*1504와 HLA-DQB1*0603으로 나타났다. 결론적으로 *P. gingivalis* 열충격단백60은 제 2급 주요조직적합체-제한적으로 분해되고 전달되었으며 이 단백질이 공통적인 T-세포 및 B-세포 epitope를 가지면서 동시에 인체 열충격단백60과 교차성 B-세포 epitope을 가지면서 동맥경화증의 면역조절기능에 관여한다고 볼 수 있다.

주요어 : *Porphyromonas gingivalis*, heat shock protein, epitope mapping, atherosclerosis