Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 240–245

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01339.x

Identification of immunoreactive epitopes of the *Porphyromonas gingivalis* heat shock protein in periodontitis and atherosclerosis

J. Choi[1], S-Y. Lee[2], K. Kim[3], B-K. Choi[4]

[1]Department of Periodontology, School of Dentistry, Pusan National University, Yangsan City, South Korea, [2]Department of Biochemistry, School of Medicine, Pusan National University, Yangsan City, South Korea, [3]Department of Pharmacology, School of Medicine, Pusan National University, Yangsan City, South Korea and [4]Department of Oral Microbiology and Immunology, School of Dentistry, Seoul National University, Seoul, South Korea

Choi J, Lee S-Y, Kim K, Choi B-K. Identification of immunoreactive epitope of Porphyromonas gingivalis *heat shock protein peptide in periodontitis and atherosclerosis. J Periodont Res 2011; 46: 240–245. © 2011 John Wiley & Sons A/S*

Background and Objective: Heat shock protein 60 (HSP60) of *Porphyromonas gingivalis*, a major periodontal pathogen, might be a trigger molecule linking infectious periodontitis and autoimmune atherosclerosis. The aim of this study was to identify the peptide specificity of anti-*P. gingivalis* HSP60 monoclonal antibodies and their cross-reactivity with bacterial and human HSPs. Their specific immunoreactivity to periodontal or atherosclerotic lesions was also investigated.

Methods: Twenty patients with chronic periodontitis and 20 atherosclerosis patients who had undergone surgical intervention for atheromatous plaques with evidence of ongoing periodontal disease, were selected. Synthetic peptide 19 ((TLVVNRLRGSLKICAVKAPG)-specific T-cell lines were established from inflamed gingiva and atheromatous plaque and the phenotypes and cytokine profiles were characterized.

Results: Thirty per cent of periodontitis patients and 100% of atherosclerosis patients reacted positively to cross-reactive peptide 19 from both *P. gingivalis* and human HSP60. The peptide 19-specific T-cell lines demonstrated the phenotype characteristic of helper T cells ($CD4^+$) but did not express CD25 or FOXP3. The interleukin-10 levels were elevated significantly in the peptide 19 T-cell line.

Conclusion: Synthetic peptide 19 of *P. gingivalis* HSP60 is an immunoreactive epitope in the periodontitis–atherosclerosis axis.

Jeomil Choi, DDS, PhD, Department of Periodontology, School of Dentistry, Pusan National University Yangsan Campus, Beomeo-ri, Yansan City, Gyeongsannam-Do 626-870, South Korea
Tel: +82 55 360 5200
Fax: +82 55 360 5194
e-mail: jrapa@pusan.ac.kr

Key words: *Porphyromonas gingivalis*; heat shock protein; periodontitis; atherosclerosis; trigger molecule

Accepted for publication November 14, 2010

A new, emerging concept that elevated levels of inflammatory biomarkers in periodontal patients might contribute to the perpetuation of atherosclerotic cardiovascular disease was announced simultaneously by two official Academies (1). Recently, heat shock protein (HSP) of *Porphyromonas gingivalis*, a major periodontal pathogen, was suggested to be a trigger molecule linking periodontitis as an infectious disease with atherosclerosis as an autoimmune disease (2–6). The pivotal role of *P. gingivalis* HSP60 in the immunopathogenic mechanism of both periodontitis and atherosclerosis has been demonstrated in the context of immunodominant T- and/or B-cell epitopes (7–10). A *P. gingivalis* HSP60 vaccine was reported to successfully reduce the level of alveolar bone loss in experimental periodontitis as a polymicrobial infection (11), and an anti-*P. gingivalis* HSP60 serum manifested cross-species

recognition to exert an opsonophagocytic function against multiple periodontopathogenic bacteria (12). However, concerns have been raised about use of the HSP60 molecule as a periodontal vaccine because of the potential for autoimmune reactions as a result of the high sequence homology of bacterial HSP with the human self-antigen.

Therefore, identification of a specific cross-reactive HSP peptide is essential for providing cross-species protection in periodontal disease as a polymicrobial infection (13). The same is true for identifying a specific antigenic region in the HSP peptide through molecular mimicry that might mobilize antigen-specific regulatory T cells to suppress the periodontitis-triggered autoimmune reaction in atherosclerosis (2,14,15).

Monoclonal hybridoma technology was adopted to clone the anti-*P. gingivalis* HSP60 monoclonal antibody (mAb) that manifests either monospecificity or polyspecificity to exogenous bacterial HSPs or to indigenous human self-HSP60 at the molecular level (16). This is because determining the epitope specificity of these mAbs might clarify the molecular mechanisms through which exogenous bacterial HSP triggers the development of autoimmune diseases (i.e. atherosclerosis, diabetes mellitus or rheumatoid arthritis).

The aims of this study were to identify (i) the specific peptide recognized by anti-*P. gingivalis* HSP60 mAbs; (ii) the cross-reactivity of anti-*P. gingivalis* HSP60 mAbs with bacterial and human HSPs; and (iii) the patient's seroreactivity to the specific peptides and to the human HSP homolog recognized by these mAbs. The peptide-specific T-cell lines established from the periodontal or atherosclerotic lesions were also characterized.

Material and methods

Identification of specific peptide epitopes

Synthetic peptides— A total of 37 overlapping peptides (each consisting of 20 amino acids with a 5-amino acid overlapping sequence), spanning the entire *P. gingivalis* HSP60 protein sequence, were synthesized by Fmoc solid-phase peptide synthesis using ASP48S (Peptron Inc., Daejeon, South Korea), and were purified by reverse-phase high-performance liquid chromatography using a Vydac Everest C18 column (Grace Vydac, Hesperia, CA, USA). Elution was carried out with a water/acetonitrile linear gradient [10–75% (v/v) of acetonitrile] containing 0.1% (v/v) trifluoroacetic acid.

Western and dot-immunoblot analysis— Briefly, each peptide (1 μg) was subject to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride membrane or spotted onto a polyvinylidene difluoride membrane. The membrane was blocked for 30 min with 5% skim milk, the mAb in phosphate-buffered saline (PBS) was added and the membrane was incubated for a further 2 h at room temperature. After washing the membrane with PBS-Tween20 for 30 min at room temperature, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (γ-chain specific; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added and the membrane was incubated for 1 h. The membrane was then washed with PBS-Tween, tetramethylbenzidine was added and any color development was noted.

Study subjects

Twenty patients with chronic periodontitis, defined as an inflammation within the supporting tissues of the teeth with progressive attachment and bone loss (Glossary of Periodontal Terms, 4th edition, AAP 2001), without a history of atherosclerosis and 20 atherosclerosis patients who had undergone surgical intervention for atheromatous plaques with evidence of ongoing periodontal disease, were selected. Only those who demonstrated elevated serum antibody titers to *P. gingivalis* HSP60 were included. A gingival specimen was removed from the periodontal lesions during periodontal open flap debridement. Atheromatous plaque was harvested during the surgical intervention procedure. Peripheral blood was drawn by venepuncture to provide antigen-presenting cells and for serum collection. The study was approved by the Institutional Review Board of Pusan National University Hospital. Informed consent forms were obtained from the patients for the surgical procedure.

Reactivity of patient sera to epitope peptides

Five micrograms of synthetic *P. gingivalis* HSP peptide recognized by either monospecific or polyspecific mAbs was spotted onto a nitrocellulose membrane. The membrane was blocked for 30 min with 5% skim milk, individual samples of serum from each patient was tested separately and the membrane was incubated for a further 2 h at room temperature. After washing the membrane with PBS-Tween20 for 30 min at room temperature, horseradish peroxidase-conjugated mouse anti-human IgG (γ-chain specific; Jackson ImmunoResearch Laboratories) was added and the membrane was incubated for a further 1 h. The membrane was then washed with PBS-Tween, tetramethylbenzidine was added and any color development was noted.

Characterization of cross-reactive peptide-specific T-cell lines

Human T cells purified from gingival or atherosclerosis lesions were stimulated with cross-reactive peptide for 2 wk by adding the peptide and mitomycin-treated peripheral blood lymphocytes. With an alternating cycle of stimulation and resting for 2 wk, the antigen-specific T-cell lines were established and characterized by flow cytometry analysis to confirm the phenotype of regulatory T cells ($CD4^+$, $CD25^+$, $FOXP3^+$). The concentrations of cytokine [interferon-γ (IFN-γ), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β)] in the T-cell culture supernatant were measured using a standard ELISA. The concentration of cytokines in the cell culture medium was used as the control.

Table 1. The mean pocket depths and serum immunoglobulin G (IgG) titers, to *Porphyromonas gingivalis* heat shock protein 60 (HSP60), in healthy controls, patients with periodontitis and atherosclerosis and patients with ongoing periodontal disease

Parameter	Healthy control (n = 20)	Periodontitis patients (n = 20)	Atherosclerosis patients (n = 20)
Mean pocket depth (mm)	2.8 ± 0.4	5.4 ± 1.3	5.6 ± 1.1
IgG titer	102 ± 11	608 ± 34*	581 ± 41*

The ELISA unit is the serum dilution factor corresponding to an optical density of 0.5.
*Significantly higher than the control group ($p < 0.01$).

Results

Patients' profile

The mean pocket depths of patients with periodontitis and of patients with atherosclerosis and ongoing periodontal disease were 5.4 ± 1.3 and 5.6 ± 1.1 mm, respectively (Table 1). The serum titers of IgG to *P. gingivalis* HSP60 in patients with periodontitis and in patients with atherosclerosis and ongoing periodontal disease were 608 ± 34 and 581 ± 41, respectively, which were significantly higher than those of the control group ($p < 0.01$).

Identification of specific peptide epitopes

Five clones (JC5–JC9) of the polyspecific anti-*P. gingivalis* HSP60 mAbs (Fig. 1A) cross-recognized HSPs from periodontopathogenic bacteria and human HSP60 (Fig. 1A, left and middle panels, respectively). These clones recognized synthetic peptide 19 (TLVV NRLRGSLKICAVKAPG) (Fig. 2A). Three clones (JC1–JC3) demonstrating monospecificity to *P. gingivalis* HSP60 (Fig. 1B) recognized peptide 29 (TVPG GGTTYIRAIAALEGLK) of the synthetic peptide spanning the entire molecule of *P. gingivalis* HSP60 (Fig. 2B), as shown in western blotting or dot immunoblot analyses.

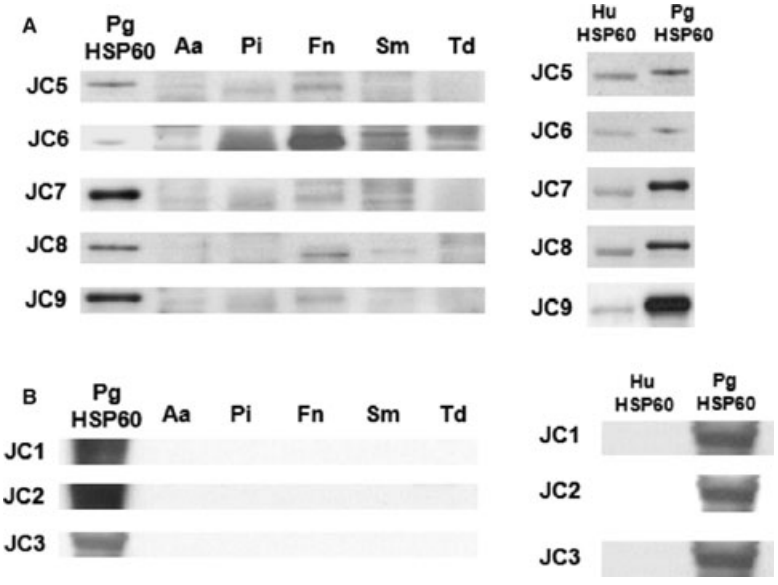

Fig. 1. Pattern of reaction of the polyreactive clones JC5–JC9 (A; left panel) and the monoreactive clones JC1–JC3 (B; left panel) of anti-*Porphyromonas gingivalis* heat shock protein 60 (HSP60) monoclonal antibodies with cognate HSP antigen or with the HSPs of five periodontopathogenic bacteria, or with the human (Hu) counterpart (A, right panel; B, right panel, respectively), demonstrated by immunoblot analysis. Aa, *Aggregatibacter actinomycetemcomitans*; Fn, *Fusobacterium nucleatum*; Pi, *Prevotella intermedia*; Sm, *Streptococcus mutans*; Td, *Treponema denticola.*

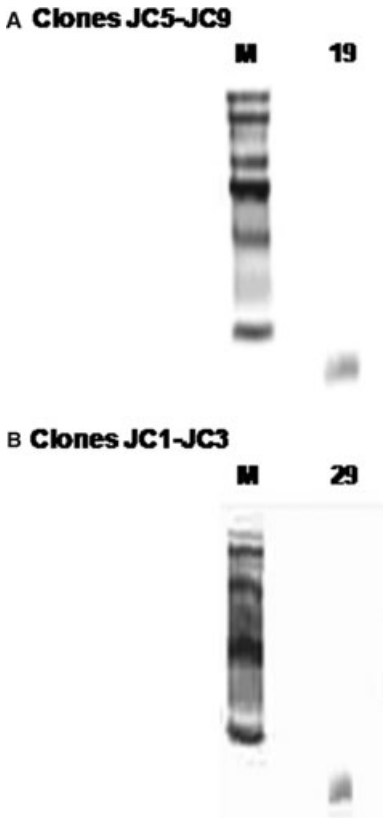

Fig. 2. The polyreactive monoclonal antibody (mAb) (A; clones JC5–JC9) recognized peptide 19 among 37 synthetic peptides spanning the whole molecule of *P. gingivalis* heat shock protein 60 (HSP60) as well as human HSP60, while the monoreactive mAb (B; clones JC1–JC3) recognized peptide 29 without responding to human HSP60, respectively, as demonstrated by western blotting.

Reactivity of patient sera to epitope peptides

P. gingivalis HSP peptide 19, which was identified by the cross-reactive mAb, was recognized by sera from each of the 20 atherosclerosis patients, whereas only six out of 20 periodontitis patients showed a positive reaction to this peptide. None of the periodontitis or atherosclerosis patient sera responded to peptide 29, which had been recognized by the monospecific mAb (Fig. 3)

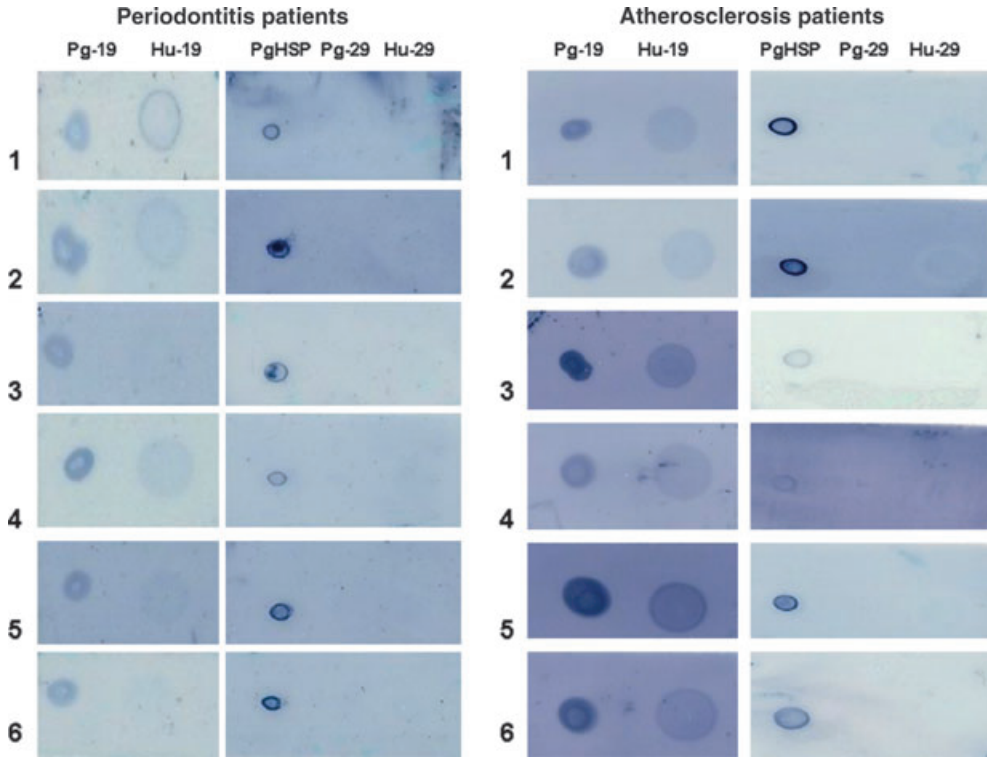

Fig. 3. Porphyromonas gingivalis heat shock protein (HSP) peptide 19 (Pg-19) and its human homolog (Hu-19) were recognized by serum from each of 20 atherosclerosis patients, whereas serum from only six of 20 periodontitis patients showed a positive reaction to the peptide. No serum from any periodontitis or atherosclerosis patients responded to peptide 29 from either *P. gingivalis* HSP (Pg-29) or human HSP (Hu-29). Peptide 29 has been recognized by a monospecific monoclonal antibody (mAb). Six randomly selected blot images are depicted (serum dilution 1 : 200).

Characterization of cross-reactive peptide-specific T-cell lines

The cross-reactive peptide 19-specific T-cell line, established from gingival or atherosclerotic lesions, demonstrated a T-cell phenotype characteristic of helper T cells ($CD4^+$) (Fig. 4). However, none of the T-cell lines established was positive for the phenotypes characteristic of regulatory T cells (CD25 or FOXP3). The peptide 19-specific T-cell line produced a significantly higher amount of IL-10 than the control supernatant in both the periodontitis and atherosclerosis patients, whereas the levels of IFN-γ and TGF-β were not elevated (Fig. 4).

Discussion

Simply comparing the sequence homology or mapping the immunodominant epitopes might provide only limited information as to which peptide of *P. gingivalis* HSP would cross-react with the human HSP peptide within the gingival lesion or arterial wall. To circumvent these underlying obstacles, monoclonal hybridoma technology was used to screen candidate peptides that exhibit either monospecificity or polyspecificity to the HSP of exogenous bacteria or to the indigenous human self-HSP at the molecular level (16,17). The concept stems from the polyreactive nature of the antibody to pathogen-associated molecular patterns, such as HSP, lipopolysaccharide or phosphorylcholine (18–20).

Eight clones were obtained from a mouse hybridoma producing a panel of monoclonal IgGs to *P. gingivalis* HSP60. Despite the monoclonality of the hybridoma cell lines, five showed an identical pattern of polyreactivity with the cognate HSP antigen, other bacterial HSPs, as well as a human homolog, whereas three demonstrated monoreactivity only to the cognate HSP60 molecule. Therefore, the polyreactive mAb, which is characteristic of the host defense mechanisms against the pattern-associated molecular pattern, was validated (18–20).

In an attempt to identify the specific peptide epitope recognized by these mAbs, 37 synthetic peptides (20-mer, overlapping by five amino acids), spanning the entire molecule of *P. gingivalis* HSP60, were synthesized. A monoreactive mAb to *P. gingivalis* HSP60 recognized peptide 29 (TVPGGGTT YIRAIAALEGLK), whereas the polyreactive mAb recognized peptide 19 (TLVVNRLRGSLKICAVKAPG). *P. gingivalis* HSP peptide 19, which was identified by a cross-reactive mAb, was recognized by serum from each patient with atherosclerosis. This is strongly indicative of a link molecule triggering atherosclerosis by a periodontal infection and is supported further by the observation that serum from each patient with atherosclerosis reacted to peptide 19 from *P. gingivalis* HSP60 and human HSP60 but not to peptide 29.

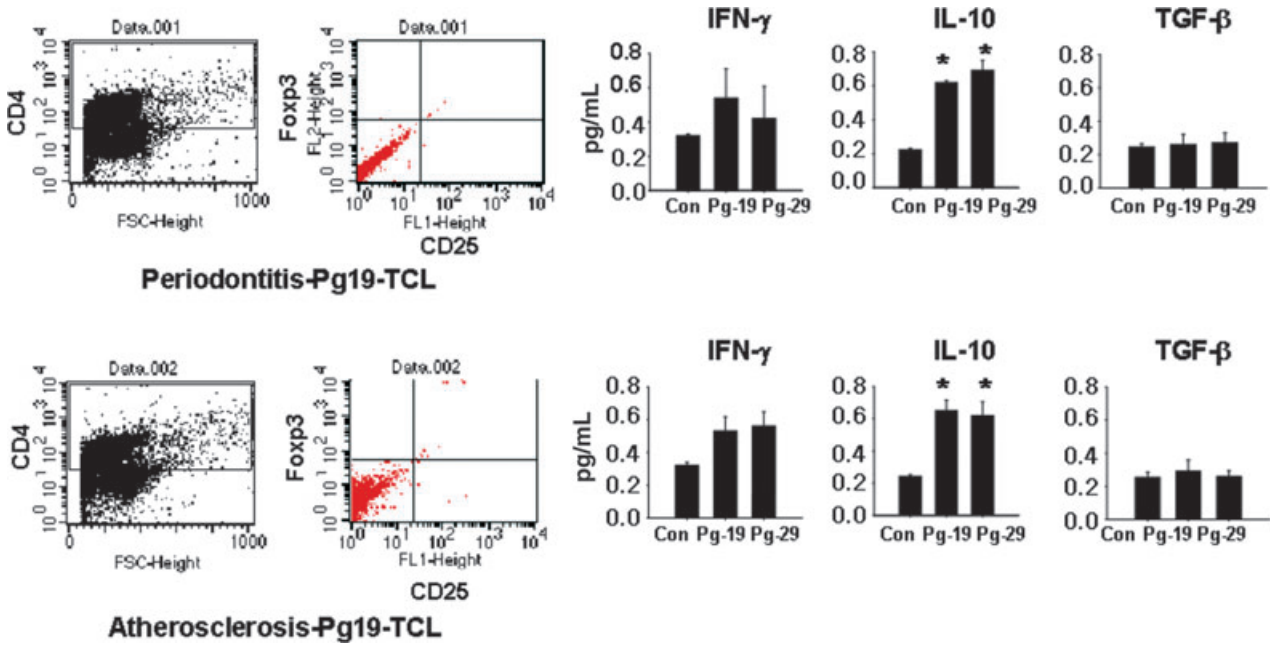

Fig. 4. Cross-reactive peptide 19-specific T-cell lines established from gingival (periodontitis-Pg19-TCL) or atherosclerotic (atherosclerosis-Pg19-TCL) lesions demonstrate a phenotype characteristic of helper T cells ($CD4^+$) without expressing FOXP3 in flow cytometric analysis. The levels of interleukin-10 (IL-10) were significantly elevated ($*p < 0.05$) in peptide 19-specific T-cell lines (Pg-19) in periodontitis and atherosclerosis patients; however, the levels of interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were not increased. Peptide 29-specific T-cell lines demonstrated a similar phenotypic profile (data not shown) with a comparable amount of cytokines produced. Pg-19, *Porphyromonas gingivalis* heat shock protein (HSP) peptide 19; Pg-29, *Porphyromonas gingivalis* heat shock protein (HSP) peptide 29.

Interestingly, based on our previous studies and on the findings of another report, peptide 19 has been identified as an immunodominant T- and B-cell epitope in both periodontitis and atherosclerosis patients (9,10,21,22), and as a common B-cell epitope in atherosclerosis patients (9), whereas peptide 29 is a B-cell epitope in periodontitis (21) and a T- and B-cell epitope in atherosclerosis patients (22). Further experiments are needed to determine if immunization with peptide 19 or adoptive transfer of a specific T-cell line would accelerate the development of atherosclerosis.

Identifying a defined cross-reactive HSP peptide is essential for providing cross-species protection in periodontal disease as a polymicrobial infection (13). HSP60 is a molecular target for the T-cell immune response in the periodontitis–atherosclerosis link (4,5,9,14). In the present study, the peptide 19-specific T-cell line demonstrated a phenotype characteristic of a helper T-cell (i.e. it was $CD4^+$), without expressing CD25 or FOXP3, but secreted a significantly higher amount of IL-10. However, the levels of IFN-γ or TGF-β were not elevated. Within the limits of this study, the cytokine profile of the T-cell line could not be delineated because the regulatory T cell developed by either peptide 19 (poly-reactive) or peptide 29 (mono-reactive) could not be observed. It is possible that peptides 19 and 29, both being the immunodominant B-cell epitopes in periodontitis and atherosclerosis (9,10,21,22), could not stimulate regulatory T cells. Screening is currently underway for epitopes that can stimulate T lymphocytes exclusively without being B-cell epitopes.

Future studies should examine whether a tailored antigen from the HSP peptide through molecular mimicry might mobilize antigen-specific regulatory T cells to suppress a periodontitis-triggered autoimmune reaction in atherosclerosis (4,5). Several studies have examined the regulatory T cells involved in the immunoregulatory mechanisms in periodontal lesions (23) and atherosclerosis (2,4,5,14,15).

In conclusion, peptide 19 (TLVV NRLRGSLKICAVKAPG) of *P. gingivalis* HSP60 is an immunoreactive epitope in periodontal infection-triggered atherosclerosis. This strategy can be expanded to identify the candidate peptide molecules linking periodontal–systemic autoimmune diseases (i.e. atherosclerosis, diabetes mellitus or rheumatoid arthritis) triggered by exogenous bacterial HSP (2).

Acknowledgements

The present study was supported by grants from the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea A080391. We are grateful to Dr Ishihara, Tokyo Dental College, for providing *Treponema denticola* culture. We also express our deep appreciation to Professor Goran K. Hansson, Karolinska Institute, Sweden, for his critical review and valuable comments. The support of Professors Eunyup Lee, Chulhoon Chang and Hyunghoi Kim, in the

process of collecting blood samples, is appreciated.

References

1. Friedewald VE, Kornman KS, Beck JD *et al*. The American Journal of Cardiology and Journal of Periodontology Editors' Consensus: periodontitis and atherosclerotic cardiovascular disease. *J Periodontol* 2009;**80:**1021–1032.
2. Sherer Y, Shoenfeld Y. Mechanisms of disease: atherosclerosis in autoimmune diseases. *Nat Clin Pract Rheumatol* 2006;**2:**99–106.
3. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol* 2005;**5:**318–330.
4. van Eden W, Wick G, Albani S, Cohen I. Stress, heat shock proteins, and autoimmunity. *Ann NY Acad Sci* 2007;**1113:**217–237.
5. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* 2006;**6:**508–519.
6. Rajaiah R, Moudgil KD. Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev* 2009;**8:**388–393.
7. Choi J, Jung SW, Kim SJ, Kim SJ. Establishment of *Porphyromonas gingivalis*-specific T cell lines from atherosclerosis patients. *Oral Microbiol Immunol* 2001;**16:**316–318.
8. Choi JI, Chung SW, Kang HS, Rhim BY, Kim SJ, Kim SJ. Establishment of *Porphyromonas gingivalis* heat-shock-protein-specific T-cell lines from atherosclerosis patients. *J Dent Res* 2002;**81:**344–348.
9. Choi JI, Chung SW, Kang HS *et al*. Epitope mapping of *Porphyromonas gingivalis* heat-shock protein and human heat-shock protein in human atherosclerosis. *J Dent Res* 2004;**83:**936–940.
10. Choi JI, Kang HS, Park YM, Kim SJ, Kim US. Identification of T-cell epitopes of *Porphyromonas gingivalis* heat-shock-protein 60 in periodontitis. *Oral Microbiol Immunol* 2004;**19:**1–5.
11. Lee JY, Yi NN, Kim US, Choi JS, Kim SJ, Choi JI. *Porphyromonas gingivalis* heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontopathogenic bacteria. *J Periodont Res* 2006;**41:**10–14.
12. Choi JI, Choi KS, Yi NN, Kim US, Choi JS, Kim SJ. Recognition and phagocytosis of multiple periodontopathogenic bacteria by anti-*Porphyromonas gingivalis* heat-shock protein 60 antisera. *Oral Microbiol Immunol* 2005;**20:**51–55.
13. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005;**6:**353–360.
14. Wick G, Perschinka H, Millonig G. Atherosclerosis as an autoimmune disease: an update. *Trends Immunol* 2001;**22:**665–669.
15. Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T cell responses: potential role in the control of atherosclerosis. *Curr Opin Lipidol* 2005;**16:**518–524.
16. Ha JA, Choi J. Monoclonal antibody to *Porphyromonas gingivalis* heat-shock protein. *J Dent Res* 2010;**89** (Spec Iss)**:** abstract number 2631.
17. Choi J, Chung SW, Lee SY, Kim KH, Choi BK. Immunoreactivity of poly-specific peptide from *Porphyromonas gingivalis* heat shock protein. *J Dent Res* 2010;**89** (Spec Iss)**:** abstract number 2716.
18. Lee J, Suh J, Choi J. B-1 cell-derived monoclonal antibodies and costimulatory molecules. *J Surg Res* 2009;**154:**293–298.
19. Notkins AL. Polyreactive antibodies and polyreactive antigen-binding B(PAB) cells. *Curr Top Microbiol Immunol* 2000;**252:**241–249.
20. Zhou ZH, Tzioufas AG, Notkins AL. Properties and function of polyreactive antibodies and polyreactive antigen-binding B cells. *J Autoimmun* 2007;**29:**219–228.
21. Maeda H, Miyamoto M, Kokeguchi S *et al*. Epitope mapping of heat shock protein 60(GroEL) from *Porphyromonas gingivalis*. *FEMS Immunol Med Microbiol* 2000;**28:**219–224.
22. Park JG, Kim SJ, Choi JI. Epitope specificity of *Porphyromonas gingivalis* heat shock protein for T-cell and/or B-cell in human atherosclerosis. *J Korean Acad Periodontol* 2003;**33:**179–191.
23. Okui T, Ito H, Honda T, Amanuma A, Yoshie H, Yamazaki K. Characterization of CD4+ FOXP3+ T-cell clones established from chronic inflammatory lesions. *Oral Microbiol Immunol* 2008;**23:**49–54.