J Periodont Res 2006; 41; 10–14

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00832.x

Porphyromonas gingivalis heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontopathogenic bacteria

Ju-Youn Lee, Ni-Na Yi, Ueon-Suk Kim, Jeoung-Soon Choi, Sung-Jo Kim, Jeom-Il Choi*
Department of Periodontology, School of Dentistry and Research Institute for Oral Biotechnology Pusan National University, Pusan, Korea

Lee J-Y, Yi N-N, Kim U-S, Choi J-S, Kim S-J, Choi J-I: Porphyromonas gingivalis *heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontopathogenic bacteria. J Periodont Res 2006; 41: 10–14. © Blackwell Munksgaard 2005*

Objectives: Heat shock protein (HSP) can be utilized as a vaccine to cross-protect against multiple pathogenic species. The present study was performed to evaluate *Porphyromonas gingivalis* heat shock protein 60 (HSP60) as a vaccine candidate to inhibit multiple bacteria-induced alveolar bone loss.

Material and methods: Recombinant *P. gingivalis* HSP60 was produced and purified from *P. gingivalis GroEL* gene. Rats were immunized with *P. gingivalis* HSP60, and experimental alveolar bone loss was induced by infection with multiple periodontopathogenic bacteria.

Results: There was a very strong inverse relationship between postimmune anti-*P. gingivalis* HSP immunoglobulin G (IgG) levels and the amount of alveolar bone loss induced by either *P. gingivalis* or multiple bacterial infection ($p = 0.007$). Polymerase chain reaction data indicated that the vaccine successfully eradicated the multiple pathogenic species.

Conclusions: We concluded that *P. gingivalis* HSP60 could potentially be developed as a vaccine to inhibit periodontal disease induced by multiple pathogenic bacteria.

*Jeom-Il Choi, Department of Periodontology, School of Dentistry, Pusan National University, 1–10, Ami-Dong, Seo-Ku, Pusan 602–739, Korea
Tel: +82 51 240 7460
Fax: +82 51 243 7606
e-mail: jrapa@pusan.ac.kr

Key words: experimental periodontitis; heat shock protein; periodontal vaccine; *Porphyromomas gingivalis*

Accepted for publication May 12, 2005

Animal experiments strongly suggest that severity of bacteria-induced periodontitis can be minimized by immunization with whole cells or antigens from the periodontopathogenic bacteria (1–5). However, several issues should be addressed pertinent to the development of a sophisticated vaccine against human periodontitis (6, 7). First, human periodontal disease is not caused by a single pathogen. Currently seven to 10 bacterial candidates have been claimed to be putative periodontal pathogens (8). The multiplicity of pathogenic organisms indicates that vaccine design against periodontitis is very complex (9). Second, bacterial whole cells or crude extract preparation for vaccination is not desirable because the antigenic determinants of bacteria potentially possess a high risk of cross-reactivity with human counterparts.

Heat shock protein (HSP) shares a high sequence homology among periodontopathic bacteria (10), which can be utilized as a vaccine to cross-protect against multiple pathogenic species. Within the context of the strategy to develop a cross-protective vaccine, we

have initially reported that *Porphyromonas gingivalis* HSP cross-reacted with the heat-induced proteins of six periodontopathogenic bacterial species and demonstrated the high opsonophagocytosis function against the six target bacteria (11). In the present study, we have extended our investigation to evaluate the vaccine efficacy of *P. gingivalis* HSP on bacteria-induced alveolar bone loss in rats.

Material and methods

Growth and maintenance of bacterial strains

Porphyromonas gingivalis 381, *Prevotella intermedia* ATCC 25611 and *Fusobacterium nucleatum* ATCC 10953 were grown in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml). *Actinobacillus actinomycetemcomitans* ATCC 33384 was grown in brain heart infusion broth supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml). *Tannerella forsythia* ATCC 43037 was grown in peptone-yeast extract medium with horse serum. Freeze-dried culture of *Treponema denticola* ATCC 33520 was a gift from Dr Ishihara (Tokyo Dental College, Chiba, Japan). Bacterial cultures were harvested, washed in phosphate-buffered saline, and finally resuspended in prereduced Ringer solution to maintain viability of anaerobic organisms. Either 5×10^8 cells of *P. gingivalis* alone or 1×10^8 cells of each bacteria in a mixed form were used for the infection of each rat.

Immunization of mouse with recombinant *Porphyromonas gingivalis* heat shock protein 60

Recombinant *P. gingivalis* HSP60 (rHSP60) was produced and purified from *P. gingivalis GroEL* gene (12), and the purity was verified as previously described (13, 14). Twelve Sprague-Dawley rats maintained in a specific pathogen-free breeding facility were initially immunized subcutaneously with *P. gingivalis* rHSP60 (5 μg) in complete Freund's adjuvant followed by two injections of the protein in incomplete Freund's adjuvant at 2-week intervals. Rat serum samples were collected by tail bleeding prior to the first immunization and 1 week after the final immunization, and stored at 20°C until used. Twelve control rats were sham-immunized with complete and incomplete Freund's adjuvant, respectively.

Induction of alveolar bone loss by bacterial infection

On day 4 following the final immunization, each rat was injected intramuscularly with a daily dose of 20 mg of ampicillin and kanamycin for three consecutive days to suppress the endogenous bacteria. Experimental alveolar bone loss was induced by bacterial infection 3 days following antibiotic treatment. Twelve immunized rats were divided into two groups: six rats were infected with *P. gingivalis* (5×10^8) alone suspended in 2% viscosity carboxylmethyl cellulose for three consecutive days by intraoral inoculation, whereas the other six rats were infected with all the six bacterial strains (1×10^8/each organism) by the same protocol. Twelve control rats (sham-immunized) were also divided into two groups: each group was infected with either *P. gingivalis* or with mixed bacteria. Bacterial infection was repeated on three consecutive days. Six weeks following the last bacterial infection, the rats were killed by injection of an overdose pentothal sodium.

Identification of periodontopathogenic bacteria in fecal samples

Fecal samples were collected 2 days following the bacterial infections and at the time of death in three rats per group and were subjected to polymerase chain reaction (PCR) amplification of rDNA to verify that the intraoral inoculation was successful. Previously published primer pairs were selected for specific PCR amplification of 16S rDNAs of the microorganisms (15, 16). PCR amplification was performed in a thermal cycler (PE2400: Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the conventional protocol. The PCR products were visually identified by agarose gel electrophoresis.

Measurement of rat anti-*Porphyromonas gingivalis* heat shock protein 60 immunoglobulin G antibody titer

Microtiter plates were coated in triplicate with *P. gingivalis* rHSP60 (10 μg/ml) diluted in phosphate buffer. Coated plates were washed and rat serum samples serially diluted in phosphate-buffered saline/Tween were added to each well and incubated. After washing the plates, peroxidase-conjugated goat anti-rat immunoglobulin G (IgG: heavy and light chains) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added and incubated. The plates were washed and tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well for incubation followed by adding H_2SO_4 to stop the reaction. Optical densities read at 450-nm wavelength were plotted as a function of serum dilution factor and regression analysis was performed. One of the preimmune control sera was assigned an enzyme-linked immunosorbent assay (ELISA) unit of 100, and serum IgG titers of the control and test sera were calculated. Antibody titers were subjected to Student's *t*-test for statistical comparison.

Measurement of bacteria-induced alveolar bone loss

After death, rat maxillary bone was defleshed to measure the distance from the cementoenamel junction to either the buccal or palatal alveolar bone crests at five sites of the upper right first and second molars using a digital stereomicroscope (Olympus, Tokyo, Japan). Significance of differences between groups was sought by analysis of variance (ANOVA) by comparing mean buccal or palatal bone levels per rat. Furthermore, postimmune antibody titers and the mean alveolar bone level per rat were plotted and statistical significance was determined using the Spearman's rank test.

Results

Anti-*Porphyromonas gingivalis* heat shock protein 60 immunoglobulin G titers

Mean anti-*P. gingivalis* HSP60 IgG titers of the control group were 110.5 ± 24.5 and 115.6 ± 33.4 at pre- and postimmune period, respectively. In six rats infected with *P. gingivalis* alone, the values were 115.1 ± 14.1 and 385.1 ± 15.8, respectively. In the other six rats infected with mixed bacteria, these values were 121.4 ± 30.6 and 873.3 ± 61.7, respectively (Table 1). Postimmune anti-*P. gingivalis* HSP60 IgG titers of rats infected with either *P. gingivalis* alone or with mixed bacteria were significantly elevated when compared with the preimmune and the control group titers ($p < 0.05$, $p < 0.005$, respectively).

Levels of alveolar bone

Mean buccal or palatal alveolar bone levels of the *P. gingivalis*-infected control group were 0.53 ± 0.13 mm and 0.73 ± 0.09 mm, respectively, whereas those of the experimental group were 0.45 ± 0.14 mm and 0.64 ± 0.13 mm, respectively (Table 2). The differences in the mean buccal bone levels were statistically significant between the two groups ($p < 0.014$). Mean buccal and palatal alveolar bone levels of the mixed-infected control group were 0.52 ± 0.14 mm and 0.77 ± 0.17 mm, respectively, whereas those of the experimental group were 0.42 ± 0.11 and 0.63 ± 0.13, respectively. The differences in the mean buccal bone levels were significant between the two groups ($p < 0.016$). Palatal bone levels did not show any statistically significant differences in either group.

Association of postimmune anti-*Porphyromonas gingivalis* immunoglobulin G titers and levels of alveolar bone

Linear regression analysis was performed to evaluate the correlation between the postimmune anti-*P. gingivalis* HSP60 titer and the mean alveolar bone level per rat. The inverse relationship between the two parameters was highly significant by Spearman's rank test ($p = 0.006$, Fig. 1).

Polymerase chain reaction identification of bacteria in fecal samples

The bacterial species in fecal samples were identifiable by PCR in the rats infected with either *P. gingivalis* alone or mixed bacteria at 2 days following the infection (Fig. 2). At the completion of the experiment, we could not identify any bacterial species in the fecal samples of the experimental group. However, *T. forsythia* was not identifiable in any samples.

Discussion

There are several antigenic determinants that share a high sequence homology among periodontopathic bacteria and that might be utilized for a vaccine to cross-protect against the multiple pathogenic species. These antigens include, but are not limited to, phosphorylcholine (17), capsular polysaccharide (18), and heat shock protein (HSP) (10). We excluded the use of phosphorylcholine because it has not been identified in *P. gingivalis*. Capsular polysaccharide was not included in our study as it is not an inducer of T-cell mediated immunity, demanding capsular polysaccharide–protein conjugation in the vaccine design (3).

We observed increased alveolar bone resorption in rats induced by *P. gingivalis* infection, which was similar to the results observed by others (4, 5). However, no study has evaluated the *in vivo* vaccine efficacy against multiple periodontopathogenic species using an animal model. Therefore, we have extended our previous *in vitro* observation that *P. gingivalis* HSP could be potentially used as a periodontal vaccine against multiple periodontopathogenic bacteria (19). In the present study, we used a rat model to evaluate *in vivo* the vaccine efficacy of *P. gingivalis* HSP in inhibiting alveolar bone loss induced by multiple bacteria after their colonization in the oral cavity. Anti-*P. gingivalis* HSP IgG antibody titers were significantly

Table 1 . Anti-Porphyromonas gingivalis heat shock protein 60 immunoglobulin G antibody titers of the control and the experimental groups at each experimental period (mean ± SD)

Group	Preimmune	Postimmune	*p*-value
Control ($n = 12$)	110.5 ± 24.5	115.6 ± 33.4	NS
Experimental 1 ($n = 6$, *P. gingivalis* alone)	115.1 ± 14.1	385.1 ± 15.8[a]	< 0.05
Experimental 2 ($n = 6$, mixed bacteria)	121.4 ± 30.6	873.3 ± 61.7[b]	< 0.005

[a,b]Antibody titers were significantly higher than the preimmune values and the control group ($p < 0.05$, $p < 0.005$, respectively).

Table 2 . Alveolar bone levels (cementoenamel junction to alveolar crest) of the control and the experimental groups measured using a digital stereomicroscope (mean value in mm ± SD)[a]

	Porphyromonas gingivalis alone			Mixed bacteria		
Infection groups	Control $n = 6$	Immunization $n = 6$	*p*-value[b]	Control $n = 6$	Immunization $n = 6$	*p*-value[c]
Buccal	0.53 ± 0.13	0.45 ± 0.14	0.014	0.52 ± 0.14	0.42 ± 0.11	0.016
Palatal	0.73 ± 0.09	0.64 ± 0.13	0.088	0.77 ± 0.17	0.63 ± 0.13	0.150

[a]Mean value and standard deviation from six mice per each group.
[b,c]Difference of buccal bone levels were highly significant between experimental and control groups when tested by ANOVA.

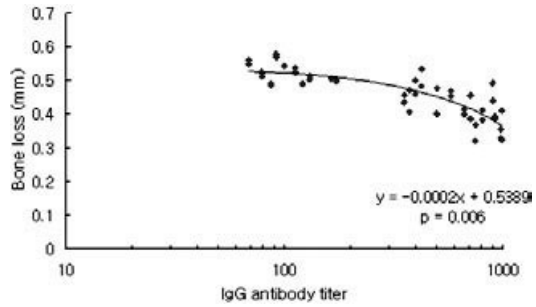

Fig. 1. Scatter plot of alveolar bone levels and anti-*Porphyromonas gingivalis* heat shock protein immunoglobulin G (IgG) antibody titers demonstrating the strong inverse correlation between the two parameters in the rats ($p = 0.006$ by Spearman's rank test).

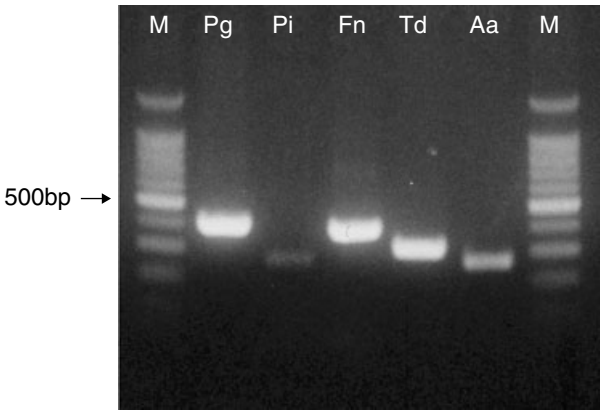

Fig. 2. Photographic features of polymerase chain reaction (PCR) products for each target bacterium identified in the fecal samples from the control or experimental groups of rats 2 days following of intraoral inoculation of the mixed bacteria (M: molecular weight marker; Pg: *Porphyromonas gingivalis*; Pi: *Prevotella intermedia*; Fn: *Fusobacterium nucleatum*; Td: *Treponema denticola*; Aa: *Actinobacillus actinomycetemcomitans*).

elevated when compared with the preimmune stage. However, there was considerable variability in postimmune antibody titer between rat groups before they were infected with either *P. gingivalis* alone or mixed bacteria. The reason is not clear; however, postvaccine variability in immune response may not be an unusual phenomenon *in vivo*. The fecal bacterial species identified by PCR suggested that the intraoral colonization could be established, except for *T. forsythia* presumably due to its fastidious nature. However, we could not verify this phenomenon directly from subgingival plaque samples due to extreme difficulty in accessibility. At the end of the experiment, we could not identify any bacterial species in the fecal samples in the experimental group.

The inhibiting effect of *P. gingivalis* HSP on the bacteria-induced buccal bone loss was highly significant regardless of whether the rats were infected with *P. gingivalis* alone or with mixed periodontopathogenic species. This observation, taken together with our previous *in vitro* observations (11), strongly suggests the utilization of *P. gingivalis* HSP as a potent periodontal vaccine against multiple periodontopathogenic bacteria. Moreover, mean alveolar bone loss per rat demonstrated a very strong inverse relationship with anti-*P. gingivalis* HSP antibody titers. Therefore anti-*P. gingivalis* HSP antibody levels could potentially be a predictor for the protective capacity in bacteria-induced periodontitis.

However, T cell immune response to bacterial HSPs may be involved in the immunopathogenic mechanism of periodontal disease (20, 21) through the induction of autoimmunity targeting mammalian HSPs. We have recently identified the immunodominant T-cell epitopes of *P. gingivalis* HSP in periodontitis patients and these epitopes could potentially be developed as peptide vaccine candidates (19). Nevertheless, due to its sequence homology with human tissue components, a major concern may arise for cross-reactive T-cell immune response leading to the induction of autoimmune reaction when the total *P. gingivalis* HSP60 is used for vaccination. To avoid the possibility of cross-reactive events, one may have to develop a vaccine strategy using synthetic peptide consisting of immunodominant epitope of the bacterial HSP that is not homologous to human homologue.

Acknowledgements

We are thankful to Professor Yoji Murayama for providing *P. gingivalis GroEL* gene, Dr Ishihara for providing bacterial culture and S. W. Lee for his expertise in flow cytometry. This work was supported by a Grant #1999-2-20500-004-3 from KOSEF.

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