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T-cell clonality to *Porphyromonas gingivalis* and human heat shock protein 60s in patients with atherosclerosis and periodontitis

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Individuals with periodontitis have been reported to have a significantly increased risk of developing coronary heart disease. Several studies have demonstrated that the immune response to heat shock protein 60 (HSP60) may be involved in the pathogenesis of both atherosclerosis and chronic periodontitis. To investigate this possible link between these diseases, cellular and humoral immune responses to HSP60 in atherosclerosis patients were compared with those in periodontitis patients and healthy subjects using human and *Porphyromonas gingivalis* HSP60 (GroEL) as antigens. Antibody levels to both human and *P. gingivalis* HSP60s were the highest in atherosclerosis patients, followed by periodontitis patients and healthy subjects. Clonal analysis of the T cells clearly demonstrated the presence of not only human HSP60- but also *P. gingivalis* GroEL-reactive T-cell populations in the peripheral circulation of atherosclerosis patients. Furthermore, these HSP60-reactive T cells seemed to be present in atherosclerotic lesions in some patients. These results suggest that T-cell clones with the same specificity may be involved in the pathogenesis of the different diseases.

Key words: atherosclerosis; HSP60; periodontitis

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Although hypertension, elevated serum cholesterol, smoking, diabetes and obesity are classical risk factors for atherosclerosis (1, 2), recent epidemiologic studies have suggested a link between atherosclerosis and inflammation as a result of infection. Associations have been reported with *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus (5, 18) and dental infections, in particular periodontal disease (3).

The general hypothesis that chronic infections can contribute to the development of atherosclerosis has come from the following:

- direct effects of infectious agents on cellular components of the vessel wall;
- increased expression of cytokines, chemokines, and cellular adhesion molecules resulting in local endothelial dysfunction;
- immune responses targeted to self-proteins located in the vessel wall mediated by molecular mimicry (8).

Heat shock protein 60 (HSP60) has received much attention from cardiovascular researchers because of its unique characteristics. Despite being highly homologous between prokaryotic and eukaryotic cells, HSP60s are strongly immunogenic. It is speculated that immune responses to microbial HSP60s initiate chronic inflammatory diseases in which autoimmune responses to human HSP60 may be central to pathogenesis (13). It is now apparent that extracellular HSPs are intercellular signaling molecules that can mediate and influence a range of inflammatory responses. Bacterial and human HSP60 activate human vascular endothelial cells to express E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1, and they activate vascular endothelial cells, smooth muscle cells, and monocyte/macrophages to secrete interleukin (IL)-6 and tumor necrosis factor-α (9, 14, 15, 26). Increased expression of human HSP60 has been observed on endothelial cells (22), macro-

phages (28), and smooth muscle cells (14) in human atherosclerotic lesions. Further, anti-chlamydial HSP60 antibody isolated from patients with coronary artery disease cross-reacts with human HSP60 to mediate endothelial cytotoxicity in the presence of complement (17).

Major periodontopathic bacteria, such as *Porphyromonas gingivalis*, are reported to produce HSPs homologous to *Escherichia coli* GroEL (10, 11, 16, 19, 27). Recently, T-cell lines specific to *P. gingivalis* GroEL, with cytokine profiles of both Th1 and Th2 cells, were established from atherosclerotic lesions of periodontitis patients, suggesting that the immune response to this antigen may be involved in the pathogenesis of atherosclerosis in these patients (7). We have also previously demonstrated that the frequency of seropositivity and the antibody titer to human HSP60 and *P. gingivalis* GroEL were significantly higher in periodontitis patients compared with periodontally healthy control subjects (25). Furthermore, affinity-purified serum antibodies to human HSP60 and *P. gingivalis* GroEL cross-reacted with *P. gingivalis* GroEL and human HSP60, respectively. Taken together, these results suggest that immune responses, either specific or cross-reactive, to both human HSP60 and *P. gingivalis* GroEL may, at least in part, explain the putative causal relationship between periodontitis and atherosclerosis.

To better understand how periodontal pathogens, particularly *P. gingivalis*, contribute to or associate with atherosclerosis, we investigated the cellular and humoral immune responses to HSP60 in patients with both periodontitis and atherosclerosis and compared these responses with those occurring in patients with periodontitis but without overt atherosclerosis and those in healthy subjects without periodontitis or overt atherosclerosis.

Material and methods

Patients

The study comprised 15 patients with both atherosclerosis and periodontitis, 16 patients with periodontitis, and 10 healthy individuals. The institutional review boards of both Niigata University Graduate School of Medical and Dental Sciences and Tachikawa General Hospital approved this study. Informed consent was obtained from each patient prior to entry into the study. The periodontal status of each of the subjects was assessed as described previously (29). Briefly, the clinical attachment level and probing pocket depth were measured at six sites per tooth; subjects with chronic periodontitis were defined according to the newly defined classification (4).

Artery biopsies were obtained at surgery for thoracic or abdominal aortic aneurysms from the 15 atherosclerosis and periodontitis patients. Gingival tissues were also obtained following extraction of periodontally affected teeth from two patients with both atherosclerosis and periodontitis. The tissues were immediately frozen in liquid nitrogen and stored at –80°C until DNA and RNA separation. The presence of stenosis of coronary arteries was confirmed by selective arteriography. Characteristics of the atherosclerosis patients are presented in Table 1. The clinical profiles of the periodontitis patients and of control subjects are shown in Table 2. None of the periodontitis patients and healthy control individuals self-reported overt atherosclerotic disease at their most recent regular medical check.

Stimulation of peripheral blood mononuclear cells with HSP60

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. PBMC were suspended in RPMI1640 (Gibco, Grand Island, NY) supplemented with 10% human AB serum (C-six Diagnostics, Inc., Mequon, WI), 20 mM HEPES buffer (Flow Laboratories, Irvine, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (Flow), 2 mM glutamine (Flow) and 5×10^{-5} M 2-mercaptoethanol. As previously described (29), cells were cultured at concentrations of 3×10^5 cells/well in a 96-well culture plate (Nunc, Roskilde, Denmark) and stimulated with either recombinant human HSP60 (Stressgen Biotechnologies Corp., Victoria, Canada) or recombinant *P. gingivalis* GroEL, prepared in our laboratory, at a concentration of 10 μg/ml for 6 days in a humidified atmosphere of 5% CO_2 and air at 37°C.

RT-PCR–SSCP analysis

Total RNA from PBMC and atherosclerotic aneurysmal tissues from atherosclerosis patients ($n = 15$) were separated by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. We also analyzed gingival tissues from two atherosclerosis patients for T-cell

Table 1. Characteristics of the atherosclerotic patients

Age	70.1 ± 7.0
Sex (male : female)	13 : 2
Total cholesterol (mg/dl)	172.8 ± 25.2 (130–219)
HDL-cholesterol (mg/dl)	41.0 ± 10.3 (40–88)
Triglyceride (mg/dl)	129.4 ± 89.6 (35–149)
Current smoker (*n*)	7
Past smoker (*n*)	4
Non-smoker (*n*)	4
Number of affected coronary arteries	1.9 ± 1.1
CRP (mg/l)	3.0 ± 0.8
Number of patients diagnosed as diabetes mellitus	1
PD <4 mm (% sites)	65.3 ± 29.5
PD 4–6 mm (% sites)	32.0 ± 26.1
PD > 6 mm (% sites)	3.1 ± 5.0
CAL < 4 mm (% sites)	31.9 ± 28.8
CAL 4–6 mm (% sites)	44.3 ± 24.4
CAL > 6 mm (% sites)	23.8 ± 32.4
Bone loss (%)	27.3 ± 12.8

PD: Probing depth; CAL: Clinical attachment level.
Data are expressed as mean ± SD.
Normal ranges for Japanese population are indicated in parentheses.

Table 2. Clinical profile of the control subjects and periodontitis patients

	Control subjects	Periodontitis patients
Age	42.4 ± 3.8	38.9 ± 9.2
Sex (male : female)	4 : 6	8 : 8
PD < 4 mm (% sites)	94.0 ± 7.6	12.7 ± 18.3
PD 4–6 mm (% sites)	6.0 ± 7.6	38.5 ± 21.6
PD > 6 mm (% sites)	0 ± 0	48.8 ± 27.6
CAL < 4 mm (% sites)	94.0 ± 7.6	6.8 ± 10.5
CAL 4–6 mm (% sites)	6.0 ± 7.6	29.0 ± 17.6
CAL > 6 mm (% sites)	0 ± 0	64.3 ± 26.2
Bone loss (%)	6.2 ± 2.7	41.6 ± 17.5

PD: Probing depth; CAL: Clinical attachment level.
Data are expressed as mean ± SD.

clonality. The RNA samples were further purified by successive treatment with DNaseI Amplification Grade (GIBCO BRL, Gaithersburg, MD). The first strand cDNA was synthesized using M-MLV reverse transcriptase (GIBCO BRL) and 50 μM random hexanucleotides (Takara Shuzo Co., LTD., Shiga, Japan) from 2 μg of total RNA in the reaction buffer (GIBCO BRL) containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, supplemented with 0.1 M dithiothreitol and dNTP (each at 0.5 mM). The reaction mixture was incubated at 37°C for 65 min, and then heated at 65°C for 10 min.

Utilizing the 22 Vβ family-specific 5' primers coupled with Cβ 3' primer designed by Choi et al. (6), polymerase chain reaction was performed with 2.5 units of Taq DNA polymerase (Promega, Madison, WI) in a final volume of 15 μl containing 6 pmol of each primer, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and dNTP (each at 0.2 mM) in an automated DNA thermal cycler (Takara). The amplification cycle profile was as follows: denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min. The duration of both denaturation in the first cycle and extension in the last cycle was extended for 7 min. After 35 cycles of amplification, the amplified DNA was diluted (2 : 18 for atherosclerotic aneurysmal tissue, 2 : 38 for PBMC) in a denaturing solution (95% formamide, 10 mM EDTA, 0.1% Bromophenol blue, 0.1% xylenecyanol) and kept at 90°C for 2 min. The diluted samples (3 μl) were electrophoresed in non-denaturing 4% polyacrylamide gels containing 10% glycerol. The gel was run at 35 W constant power for 130 min. After electrophoresis, the DNA was transferred to Immobilon-Ny (Millipore Intertech, Bedford, MA) and hybridized with biotinylated Cβ probe (5′-A (AC)AA (GC)GTGTTCCCACCCGAGG-TCGCTGTGTT-3′), visualized after incubations with streptavidin, biotinylated alkaline phosphatase and chemiluminescent substrate system (Phototope-Star detection kit; New England Biolabs, Inc., Beverly, MA). The membrane was exposed to X-ray film (Fuji RX-U; Fuji film Co., Minamiashigara, Kanagawa, Japan) and processed. The X-ray films were photographed, and their image data were analyzed using computer software (NIHimage version 1.62). The number of distinct bands on the films was counted. To minimize the effect of film processing on the results, we determined the background density and the densest area, which were scored as 0 and 100, respectively. Only the bands scored greater than 50 were considered clonal accumulation and counted (12).

Measurement of serum antibody levels to human HSP60 and *P. gingivalis* GroEL

Serum IgG antibody levels to human HSP60 and *P. gingivalis* GroEL were determined by ELISA. Microtiter plates were coated with either recombinant *P. gingivalis* GroEL or recombinant human HSP60 (100 μl/well; 5 μg/ml in 0.05 M carbonate buffer, pH 9.6) prepared in our laboratory overnight at 4°C. After washing three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T; pH 7.4), non-specific binding sites were blocked with PBS-T containing 1% bovine serum albumin for 1.5 h at room temperature. The plates were washed with PBS-T and 100 μl of test samples diluted with blocking reagent (1 : 100) were added and incubated for 1 h at room temperature. After washing three times with PBS-T, horseradish peroxidase-conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) was added and incubated further for 1 h at room temperature. The *o*-phenylene diamine diluted to 0.5 mg/ml in 0.1 M citrate buffer containing 0.015% hydrogen peroxide was added. Color development was stopped by the addition of 4 M sulfuric acid and absorbance was read at a wavelength of 490 nm using an automated enzyme-linked immunosorbent assay (ELISA) reader (Labsystems Oy, Helsinki, Finland) and analyzed using GENESIS LITE software (Labsystems). Sera demonstrating the highest and the lowest antibody levels in our previous study (25) were used as positive controls. Negative controls consisted of sera without demonstrable antibody.

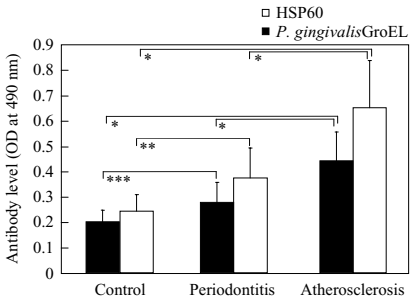


Fig. 1. Levels of IgG antibodies to human HSP60 (open column) and *P. gingivalis* GroEL (closed column) in control subjects ($n = 10$), periodontitis patients ($n = 16$) and atherosclerosis patients with periodontitis ($n = 14$). Data are expressed in mean values ± standard deviation. The serum antibody levels for antigens of atherosclerosis patients were significantly higher than those of either control subjects ($P < 0.0001$) or periodontitis patients ($P < 0.0001$). The serum antibody levels of periodontitis patients were significantly higher than those of control subjects for HSP60 ($P = 0.0039$) and *P. gingivalis* GroEL ($P = 0.009$). $^{*}P < 0.0001$; $^{**}P = 0.0039$; $^{***}P = 0.009$.

Detection of *P. gingivalis* in dental plaque and atherosclerotic lesions

The presence of *P. gingivalis* in dental plaque samples and in atherosclerotic aneurysmal tissues was analyzed by polymerase chain reaction (BML Inc., Tokyo, Japan). Subgingival plaque samples were taken from the deepest periodontal pocket in each patient using two paper points. The paper points were removed after 10 s, and placed into plastic vials. After addition of elution buffer (10 mM Tris–HCl, 1 mM EDTA), the vials were vortexed and centrifuged, and the supernatant as a template for polymerase chain reaction analysis. DNA was extracted from a piece of atherosclerotic aneurythmal tissues by the use of a DNA extraction kit (Wako Pure Chemical Industries, Inc., Osaka, Japan). Amplification was done using *P. gingivalis* species-specific primers (5′-AGGCAGCT-

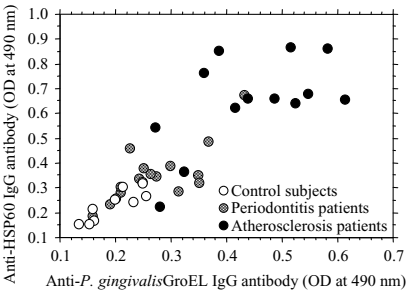
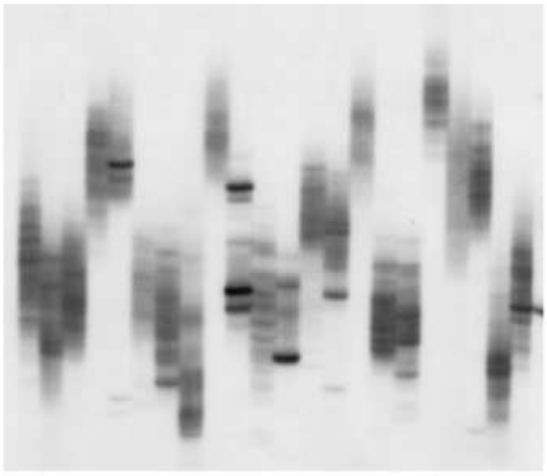


Fig. 2. Relationship between IgG antibody levels to human HSP60 and *P. gingivalis* GroEL in each group. The data are expressed as optical density at 490 nm.

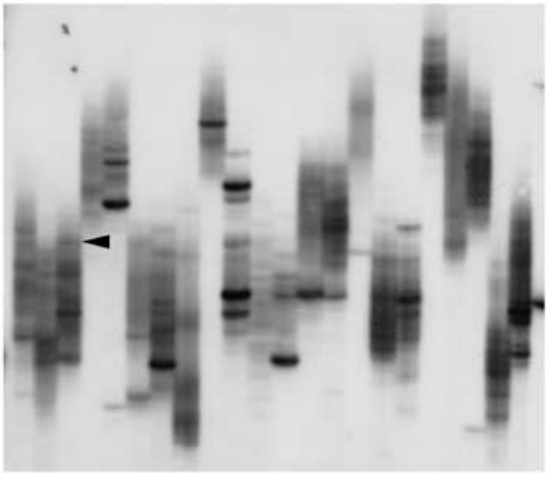
Fig. 3. T-cell clonalities of PBMC, with or without stimulation by either human HSP60 or *P. gingivalis* GroEL, atherosclerotic aneurysmal tissue and gingival tissue from an atherosclerosis patient. Photographs are representative of one atherosclerotic patient affected with periodontitis. Polymerase chain reaction products encoding T-cell receptor Vβ genes from PBMC and gingival tissue were analyzed by the SSCP method as described in Material and methods. A: Unstimulated PBMC. B: Human HSP60-stimulated PBMC. C: *P. gingivalis*-stimulated PBMC. D: Atherosclerotic aneurysmal tissue. E: Gingival tissue-derived T-cell clones. Each lane represents Vβ1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10,11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 19, 20, from left to right. The bands indicated by the arrowheads are supposed to have identical electrophoretic mobility, suggesting an identical clone.

(A)



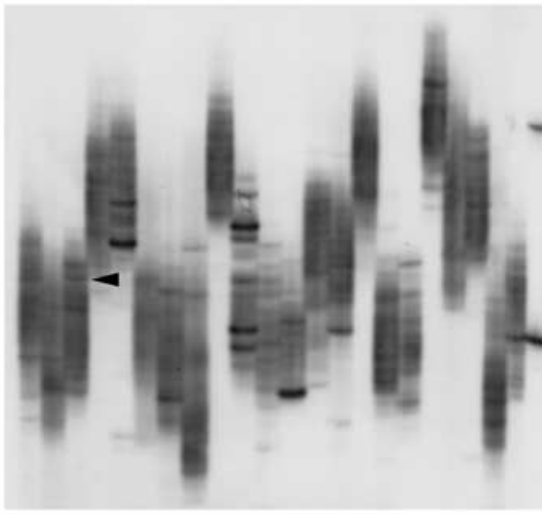
V 1 3 5.1 6 8 10 12 13.2 15 17 19
2 4 5.2 7 9 11 13.1 14 16 18 20

(B)



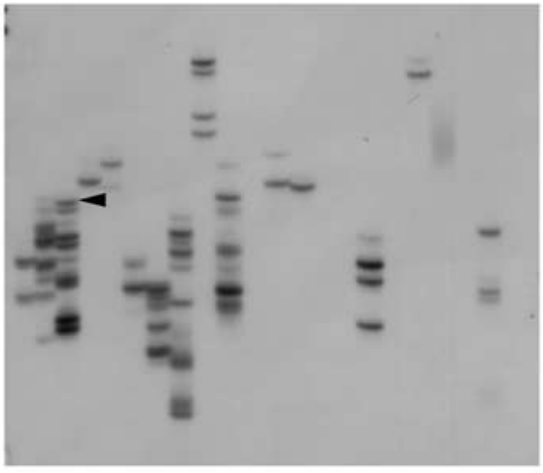
Vβ 1 3 5.1 6 8 10 12 13.2 15 17 19
2 4 5.2 7 9 11 13.1 14 16 18 20

(C)



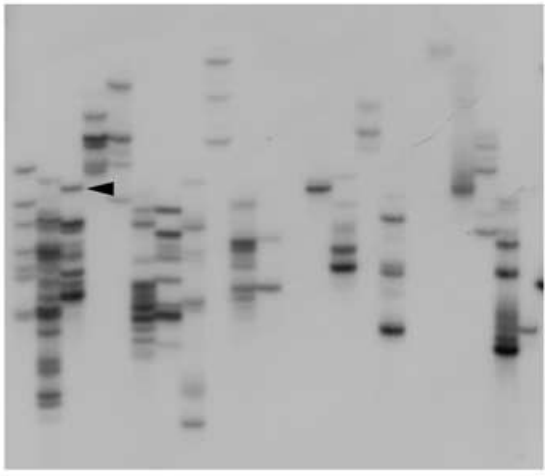
Vβ 1 3 5.1 6 8 10 12 13.2 15 17 19
2 4 5.2 7 9 11 13.1 14 16 18 20

(D)



Vβ 1 3 5.1 6 8 10 12 13.2 15 17 19
2 4 5.2 7 9 11 13.1 14 16 18 20

(E)



Vβ 1 3 5.1 6 8 10 12 13.2 15 17 19
2 4 5.2 7 9 11 13.1 14 16 18 20

TGCCATACTGCG-3′ and 5′-ACTGTT-AGCAACTACCGATGT-3′). DNA from *P. gingivalis* ATCC33277 and distilled water were used as positive and negative controls, respectively.

Statistical analysis

The unpaired *t*-test was used for data analysis. Serum antibody levels were compared between groups. The number of bands of SSCP analysis indicating T-cell clonality were compared between unstimulated, HSP60-stimulated, *P. gingivalis* GroEL-stimulated PBMC, and atherosclerotic aneurysmal tissues within the atherosclerosis patients. The correlation coefficient between HSP60 and *P. gingivalis* GroEL antibody levels in each serum sample was analyzed. A probability value <0.05 was considered statistically significant.

Results

Clinical profile of the patients

All atherosclerosis patients had moderate to severe periodontitis as well as stenosis of coronary arteries. Although diabetes is a major risk factor for atherosclerotic aneurysm and stenosis of coronary arteries, only one patient was diagnosed as having diabetes. While the levels of total cholesterol, HDL-cholesterol and triglyceride did not exceed normal ranges for the Japanese population, the mean C-reactive protein (CRP) values of the atherosclerosis patients (3.0 ± 0.8) were elevated compared to those of periodontitis patients (1.0 ± 1.5) and healthy subjects (0.9 ± 0.4).

Although the polymerase chain reaction method for the detection of bacteria is highly specific and sensitive, *P. gingivalis* was detected in only three of 15 atherosclerotic aneurysmal tissues, whereas 12 of 14 dental plaque samples harbored *P. gingivalis* (one patient was edentulous).

Serum antibody levels to human HSP60 and *P. gingivalis* GroEL

Serum antibody levels to HSP60 and *P. gingivalis* GroEL in atherosclerosis patients, periodontitis patients and healthy control subjects are shown in Fig. 1. The mean optical density values of antibody levels were 0.65 (range: 0.23–0.88) for HSP60 and 0.44 (range: 0.27–0.61) for *P. gingivalis* GroEL in atherosclerosis patients with periodontal disease. These antibody levels were significantly higher than those of healthy control subjects (mean: 0.24, range: 0.16–0.33 for HSP60, mean: 0.20, range: 0.13–0.26 for *P. gingivalis* GroEL) ($P < 0.0001$ for both HSP60 and *P. gingivalis* GroEL) and periodontitis patients (mean: 0.37, range: 0.19–0.68 for HSP60. mean: 0.28, range: 0.19–0.43 for *P. gingivalis* GroEL) ($P < 0.0001$ for HSP60 and $P < 0.0001$ for *P. gingivalis* GroEL). In addition, those in periodontitis patients were significantly higher than in control subjects ($P = 0.0039$ for HSP60 and $P = 0.009$ for *P. gingivalis* GroEL).

When the correlation between serum antibody levels to HSP60 and to *P. gingivalis* GroEL was analyzed, statistically significant correlations were found for each group (i.e. control, periodontitis and atherosclerosis patients) (Fig. 2). Interestingly, the correlation coefficient for the atherosclerosis patients was much lower than that for control subjects and periodontitis patients (0.59, 0.89 and 0.82, respectively).

SSCP analysis

Although unstimulated T cells demonstrated a smear pattern with a few bands in atherosclerosis patients (Fig. 3A), a number of distinct bands appeared after stimulation with human HSP60 and *P. gingivalis* GroEL (Fig. 3B,C). These indicate that human HSP60 and *P. gingivalis* GroEL-specific T cells existed in the peripheral blood T-cell pool. On the other hand, T cells in atherosclerotic aneurysmal tissue and gingival tissue demonstrated obvious clonal accumulation as evidenced by the number of distinct bands (Fig. 3D,E). The total number of distinct bands which reflect the clonality of the infiltrating T cells was analyzed in atherosclerosis patients. The mean total number of distinct bands in unstimulated PBMC-derived samples was 39.3 (Fig. 4). The

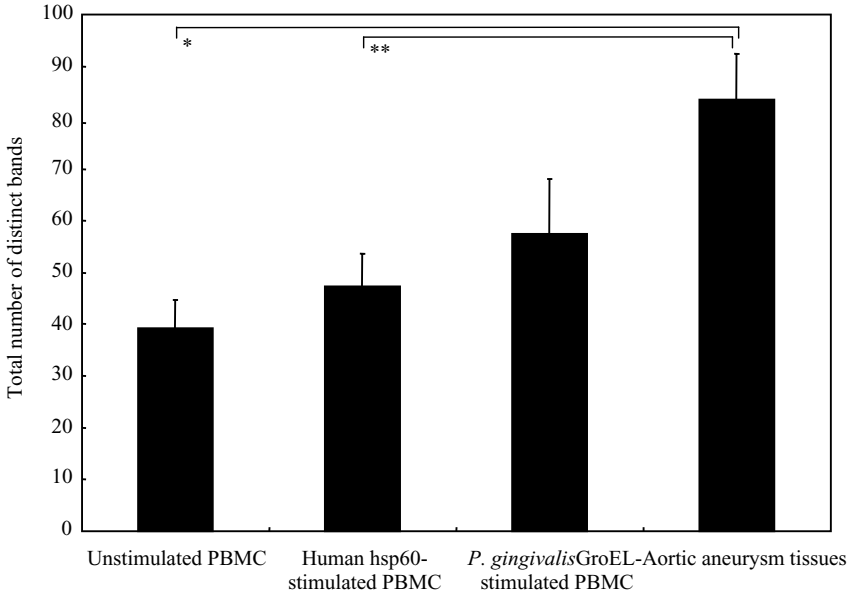


Fig. 4. Comparison of the T-cell clonality between atherosclerotic aneurysmal tissues and peripheral blood with or without stimulation. Total number of distinct bands derived from peripheral blood samples ($n = 12$) and atherosclerotic aneurysmal tissue-derived samples ($n = 12$) were counted. Three out of 15 samples were excluded from the analysis due to insufficient amount of DNA from the tissues. Data are expressed in mean values ± standard error. The number of bands derived from aortic aneurysmal tissues was significantly higher than that of unstimulated PBMC ($P = 0.0003$) and HSP60-stimulated PBMC ($P = 0.0032$). $^{*}P = 0.0003$; $^{**}P = 0.0032$.

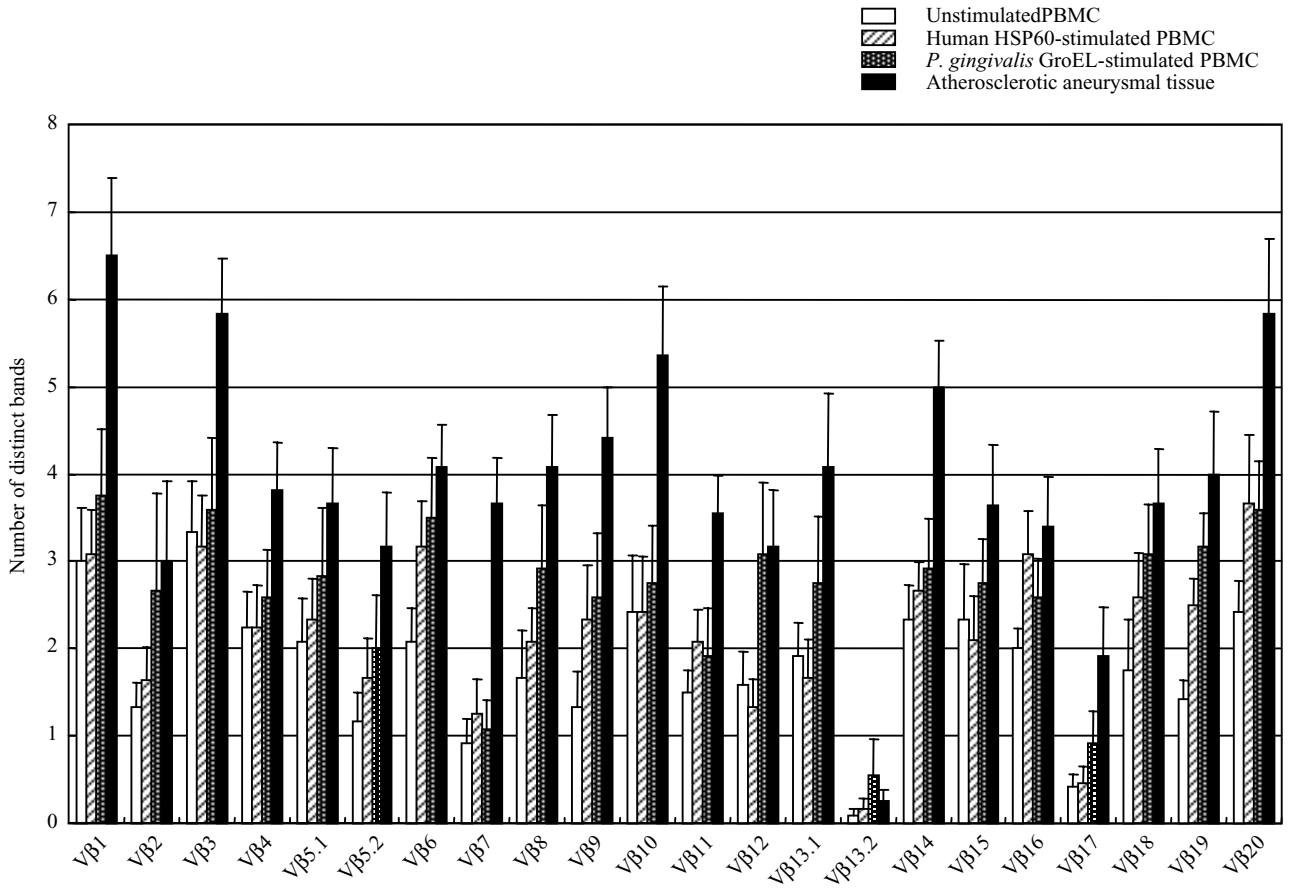


Fig. 5. Comparison of the clonality of T cells bearing each Vβ family in PBMC from the atherosclerosis patients cultured with and without stimulation and atherosclerotic aneurysmal tissues ($n = 12$). Data are expressed as mean ± standard error. Open column: unstimulated PBL. Hatched column: PBMC stimulated with HSP60. Dotted column: PBMC stimulated with *P. gingivalis* GroEL. Closed column: T cells in the atherosclerotic aneurysmal tissue.

number of bands further increased in HSP60-stimulated PBMC (47.3), *P. gingivalis* GroEL-stimulated PBMC (57.5) and atherosclerotic aneurysmal tissues (83.6), and was significantly higher in atherosclerotic aneurysmal tissue-derived samples than in unstimulated PBMC and HSP60-stimulated PBMC. A number of new distinct bands appeared after stimulation with human HSP60 and *P. gingivalis* GroEL in atherosclerosis patients. Furthermore, the emergence of new distinct bands was observed more often in the *P. gingivalis* GroEL cultures than in the human HSP60-stimulated cultures, notably in Vβs 2, 8, 12, 13.1, 17 and 19 (Fig. 5). T-cell clones expressing Vβ6 and Vβ16 were also expanded in response to HSP60 stimulation. These indicate that there is a higher number of *P. gingivalis* GroEL-specific T cells than human HSP60 in the peripheral blood of atherosclerosis patients compared with non-atherosclerotic patients.

In some cases, atherosclerotic aneurysmal tissue contained human HSP60-specific or *P. gingivalis* GroEL-specific T-cell clones, as the position of some of the newly emerged bands seemed to be identical with the bands of atherosclerotic tissue samples. Moreover, these T-cell clones seemed to exist in the gingival tissues of one of two patients from whom we could analyzed PBMC, aortic aneurysmal tissue and gingival tissue. This assumption is derived from the principle of SSCP analysis in that the electrophoretic mobility of the denatured single-strand DNA fragments can be determined by their nucleotide sequences. Therefore, the DNA fragments derived from T-cell clones bearing identical T-cell receptor β-chain would show the identical electrophoretic mobility on an SSCP gel. To confirm this, polymerase chain reaction products for the expected Vβ family were prepared from the samples stimulated with human HSP60 and/or *P. gingivalis* GroEL, atherosclerotic aneurysmal tissue-derived samples, and gingival tissue-derived samples. These were applied to adjacent lanes on the same SSCP gel only when there seemed to be distinct bands showing identical electrophoretic mobility. SSCP analysis of the expected Vβ family clearly demonstrated that peripheral blood samples stimulated with human HSP60 and *P. gingivalis* GroEL, as well as gingival tissue samples and atherosclerotic aneurysmal tissue samples, showed identical electrophoretic mobility (Fig. 6). In one patient, the identical T-cell clones were found in human HSP60-stimulated PBMC, *P. gingivalis* GroEL-stimulated PBMC, the periodontitis lesion and the atherosclerotic aneurysmal tissue.

Discussion

In the present study, antibody levels to both human HSP60 and *P. gingivalis* GroEL in atherosclerosis patients with periodontitis were significantly higher than those of periodontitis patients and healthy controls. In addition, antigen-specific T cells to human HSP60 and *P. gingivalis* GroEL were found in both peripheral blood and atherosclerotic aneurysmal tissues. As the HSP60 family of proteins

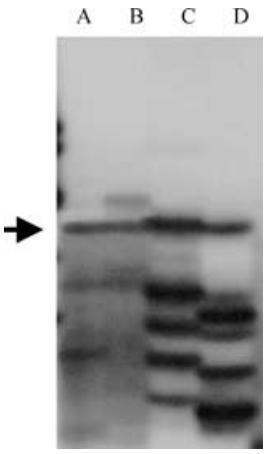


Fig. 6. Demonstration of common Vβ3 T-cell receptors in PBMC stimulated with HSPs, atherosclerotic aneurysmal tissue and gingival tissue from the atherosclerosis patient described in Material and methods as indicated by arrowhead in Fig. 3. Polymerase chain reaction products encoding T-cell receptor Vβ genes from PBMC, gingival tissue and atherosclerotic aneurysmal tissue were analyzed by the SSCP method as described in Material and methods. Arrowhead indicates identical clone. A: HSP60-stimulated PBMC. B: *P. gingivalis* GroEL-stimulated PBMC. C: Atherosclerotic aneurysmal tissue-derived tissue. D: Gingival tissue-derived T-cell clones.

appears to be phylogenetically highly conserved between human and bacterial species, immunologic cross-reactions may facilitate endothelial cell dysfunction. Although many cardiovascular researchers have thus far focused on *C. pneumoniae* and Mycobacteria, considering the homology of HSP60 between different species of bacteria, it is highly possible that the immune response to periodontopathic bacterial HSP60 may contribute to atherosclerosis by the same mechanisms as for chlamydial HSP60. In this context, we have already shown that antibodies to *P. gingivalis* GroEL in sera and gingival tissues of patients with periodontitis cross-reacted with human HSP60 (25). This indicates that anti-*P. gingivalis* GroEL antibody could react with HSP60 expressed on injured endothelial cells. As serum antibodies to HSP60 of *E. coli* and *C. pneumoniae* mediate endothelial cytotoxicity, cytotoxic activity of antibody to *P. gingivalis* GroEL is highly plausible. The reason for the elevated antibody levels in atherosclerosis patients with periodontitis compared with patients with only periodontitis may be that antibodies cross-reactive with *P. gingivalis* GroEL or human HSP60, but elicited by other bacteria such as *C. pneumonia*, might also be detected in our ELISA system. Interestingly, although antibody levels to HSP60 were significantly correlated to those to P. gingivalis GroEL in all three groups, the correlation coefficient in atherosclerosis patients was much lower than those in control subjects and periodontitis patients. This may reflect an altered humoral response to HSP60 in atherosclerosis patients. Nevertheless, elevated antibodies to HSP60 which may partly be due to periodontal infection could be a risk factor for atherosclerosis.

SSCP analysis clearly demonstrated that the peripheral T-cell pool contained antigen-specific T cells to human HSP60 and *P. gingivalis* GroEL and that oligoclonal T-cell accumulation was found in atherosclerotic aneurysmal tissue. In support of this, oligoclonal T-cell expansions were found in atherosclerotic lesions of apolipoprotein E-deficient mouse (21). In contrast, Oksenberg et al. (20) demonstrated, by using Vβ usage and CDR3 size spectratyping, that a local T-cell clonal expansion in atherosclerosis is unlikely. There is also the suggestion that more advanced human atherosclerotic plaques have a polyclonal T-cell composition (23, 24). Paulsson et al. (21) showed in their animal experiment that T-cell receptor heterogeneity was reduced in the more mature atherosclerotic plaques compared with early lesions, suggesting that a heterogeneous population of T cells is initially recruited to the forming lesion by antigenically non-specific mechanisms, followed by a selective expansion of T cells carrying specific reactivity to local antigens. Nevertheless, our study not only clearly shows clonality of the T cells in these lesions but also suggests the existence of *P. gingivalis* GroEL and HSP60 specific clones. The precise reasons for the discrepancy between our results and the data of Oksenberg et al. (20), Stemme et al. (23) and Swanson et al. (24) is unclear. One explanation could be that the stage of the disease could have been different in our study (i.e. a more mature lesion).

The number of distinct bands, which corresponds to the number of accumulated T-cell clones, of unstimulated PBMC samples was higher than that of healthy subjects and patients with only periodontitis, suggesting that the patients in the present study were exposed to recent and/or persistent antigenic challenge. While *P. gingivalis* GroEL induced greater clonal expansion of T cells in atherosclerosis patients, HSP60 was greater in periodontitis patients. Therefore, periodontitis patients with a high T-cell response to *P. gingivalis* GroEL may be at higher risk of future atherosclerosis. The number of bands expressing Vβ6, Vβ12, Vβ16 and Vβ19 increased in response to *P. gingivalis* GroEL and was similar to that of atherosclerotic aneurysmal tissue. This suggests a possible link between the immune response to *P. gingivalis* GroEL in periodontitis and in atherosclerosis. In some cases, identical T-cell clones appeared to be expanded by the stimulation with either human HSP60 or *P. gingivalis* GroEL, as the position of the newly emerged bands was identical with the different stimuli. Moreover, these T-cell clones seemed to exist in the gingival tissues as well as in atherosclerotic aneurysmal tissue. As shown in Fig. 6, a distinct band derived from T-cell clone bearing Vβ3 clearly demonstrated the identical electrophoretic mobility.

Although the presence of *P. gingivalis* GroEL-specific T cells in the atherosclerosis tissues has already been reported (7), our results show the simultaneous presence of cross-reactive T cells to HSP60 and *P. gingivalis* GroEL in periodontitis tissue and atherosclerotic aneurysmal tissue. Although functional analysis has not been carried out, these T cells probably belong to the Th1 phenotype (29). In conclusion, cross-positivity between anti-*P. gingivalis* GroEL immune response and human HSP60 expressed in stressed arteries may be related to atherogenesis. However, age differences between the atherosclerosis patients and the controls and periodontitis patients must be taken into consideration. Therefore, studies using age-matched subjects will be needed to confirm the present study. Also, other immunologic responses and genetic characteristics of the patients should be considered.

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References

1. Anderson KM, Castelli WP, Levy D. Cholesterol and mortality: 30 years of follow-up from the Framingham study. JAMA 1987: **257**: 2176–2180.
2. Assmann G, Cullen P, Schulte H. The Munster Heart Study (PROCAM): results of follow-up at 8 years. Eur Heart J 1998: **19**: A2–A11.
3. Beck JD, Pankow J, Tyroler HA, Offenbacher S. Dental infection and atherosclerosis. Am Heart J 1999: **138**: S528–S533.

4. Califano JV, Scifferle RE, Gunsolley JC, Best AM, Schenkein HA, Tew JG. antibody reactive with *Porphyromonas gingivalis* serotype K1-6 in adult and generalized early-onset periodontitis. J Periodontol 1999: **70**: 730–735.
5. Chiu B, Viira E, Tucker W, Fong IW. *Chlamydia pneumoniae*, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. Circulation 1997: **96**: 2144–2148.
6. Choi Y, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of *Staphyrococcus aureus* toxin 'Superantigens' with human T cells. Proc Natl Acad Sci USA 1989: **86**: 8941–8945.
7. Choi JI, Chung SW, Kang HS, Rhim BY, Kim SJ. Establishment of *Porphyromonas gingivalis* heat-shock-protein-specific T-cell lines from atherosclerosis patients. J Dent Res 2002: **81**: 344–348.
8. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. Circulation 1999: **100**: e20–e28.
9. Gardiero MC, de l'Ero GC, Marcatili A. Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. Infect Immun 1997: **65**: 699–709.
10. Hinode D, Yoshioka M, Tanabe S, Miki O, Masuda K, Nakamura R. The groEL-like protein from *Campylobacter rectus*: immunological characterization and interleukin-6 and -8 induction in human gingival fibroblast. FEMS Microbiol Lett 1998: **167**: 1–6.
11. Hotokezaka H, Hayashida H, Ohara N, Nomaguchi H, Kobayashi K, Yamada T. Cloning and sequencing of the groESL homologue from *Porphyromonas gingivalis*. Biochim Biophys Acta 1994: **1219**: 175–178.
12. Itoh H, Ohsawa Y, Yoshie H, Yamazaki K. Oligoclonal accumulation of T-cell clones in gingivitis and periodontitis lesions. Oral Microbiol Immunol 2002: **17**: 324–329.
13. Kiessling R, Gronberg A, Ivanyi J, et al. Role of hsp60 during autoimmune and bacterial inflammation. Immunol Rev 1991: **121**: 91–112.
14. Kol A, Bourcier T, Lichtman AH, Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest 1999: **103**: 571–577.
15. Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. J Immunol 2000: **164**: 13–17.
16. Maeda H, Miyamoto M, Hongyo H, Nagai A, Kurihara H, Murayama Y. Heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*: molecular cloning and sequence analysis of its gene and purification of the recombinant protein. FEMS Microbiol Lett 1994: **119**: 129–136.
17. Mayr M, Metzler B, Kiechl S, et al. Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of Escherichia coli and *Chlamydia pneumoniae*: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. Circulation 1999: **99**: 1560–1566.
18. Mendall MA, Goggin PM, Molineaux N, et al. Relation of *Helicobacter pylori* infection and coronary heart disease. Br Heart J 1994: **71**: 437–439.
19. Nakano Y, Inai Y, Yamashita Y, et al. Molecular and immunological characterization of a 64-kDa protein of *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol 1995: **10**: 151–159.
20. Oksenberg JR, Stavri GT, Jeong MC, Garovoy N, Salisbury JR, Erusalimsky JD. Analysis of the T-cell receptor repertoire in human atherosclerosis. Cardiovasc Res 1997: **36**: 256–267.
21. Paulsson G, Zhou X, Törnquist E, Hansson GK. Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2000; **20**: 10–17.
22. Schett G, Xu Q, Amberger A, et al. Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. J Clin Invest 1995: **96**: 2569–2577.
23. Stemme S, Rymo L, Hansson GK. Polyclonal origin of T lymphocytes in human atherosclerotic plaques. Lab Invest 1991: **65**: 654–660.
24. Swanson SJ, Rosenzweig A, Seidman JG, Libby P. Diversity of T-cell antigen receptor Vβ gene utilization in advanced human atheroma. Arterioscler Thromb 1994: **14**: 1210–1214.
25. Tabeta K, Yamazaki K, Hotokezaka H, Yoshie H, Hara K. Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. Clin Exp Immunol 2000: **120**: 285–293.
26. Ueki K, Tabeta K, Yoshie H, Yamazaki K. Self-heat shock protein 60 induces tumor necrosis factor-α in monocyte-derived macrophage: possible role in chronic inflammatory periodontal disease. Clin Exp Immunol 2002: **127**: 72–77.
27. Vayssier C, Mayrand D, Grenier D. Detection of stress proteins in *Porphyromonas gingivalis* and other oral bacteria by Western immunoblotting analysis. FEMS Microbiol Lett 1994: **121**: 303–307.
28. Xu Q, Luef G, Weimann S, Gupta RS, Wolf H, Wick G. Staining of endothelial cells and macrophages in atherosclerotic lesions with human heat-shock protein-reactive antisera. Arterioscler Thromb 1993: **13**: 1763–1769.
29. Yamazaki K, Ohsawa Y, Tabeta K, et al. Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. Infect Immun 2002: **70**: 2492–2501.