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Cross-reactivity of GroEL antibodies with human heat shock protein 60 and quantification of pathogens in atherosclerosis

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Background/aims: Chronic infections such as those caused by *Chlamydia pneumoniae* and periodontopathic bacteria such as *Porphyromonas gingivalis* have been associated with atherosclerosis, possibly due to cross-reactivity of the immune response to bacterial GroEL with human heat shock protein (hHSP) 60.
Methods: We examined the cross-reactivity of anti-GroEL and anti-*P. gingivalis* antibodies with hHSP60 in atherosclerosis patients and quantified a panel of six pathogens in atheromas.
Results: After absorption of plasma samples with hHSP60, there were variable reductions in the levels of anti-GroEL and anti-*P. gingivalis* antibodies, suggesting that these antibodies cross-reacted with hHSP60. All of the artery specimens were positive for *P. gingivalis*. *Fusobacterium nucleatum*, *Tannerella forsythia*, *C. pneumoniae*, *Helicobacter pylori*, and *Haemophilus influenzae* were found in 84%, 48%, 28%, 4%, and 4% of arteries, respectively. The prevalence of the three periodontopathic microorganisms, *P. gingivalis*, *F. nucleatum* and *T. forsythia*, was significantly higher than that of the remaining three microorganisms.
Conclusions: These results support the hypothesis that in some patients, cross-reactivity of the immune response to bacterial HSPs including those of periodontal pathogens, with arterial endothelial cells expressing hHSP60 may be a possible mechanism for the association between atherosclerosis and periodontal infection.

Key words: antibody responses; atherosclerosis; heat shock proteins; molecular mimicry; periodontal immunology

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Atherosclerotic cardiovascular disease is a leading cause of death worldwide. In recent times there has been increasing awareness that immune responses are central to atherogenesis. Chronic inflammatory periodontal disease is a significant oral health problem. *Porphyromonas gingivalis* and *Tannerella forsythia* are important organisms associated with periodontitis. Individuals with severe chronic periodontitis have been reported to have a significantly increased risk of developing cardiovascular disease including atherosclerosis, myocardial infarction and stroke (5, 12, 32, 34). Tooth loss levels, used as an indicator of cumulative periodontitis, have been associated significantly with peripheral arterial disease (14) and carotid artery plaque prevalence (6). Animal models further support the association between periodontal disease and cardiovascular disease. Murine models of atherosclerosis have demonstrated that repeated inoculation with *P. gingivalis* resulted in more advanced atherosclerotic lesions which developed more rapidly than in control mice (16, 18).

Other chronic infections by intracellular pathogens such as *Helicobacter pylori,* hepatitis A virus, herpes simplex virus

(HSV) 1, HSV 2 and cytomegalovirus have also been implicated in atherosclerosis (8, 23, 24, 30, 37, 38). Until recently, however, studies have concentrated on the effect of infection with a single pathogen. Epstein et al. (7) postulated that multiple pathogens are involved and that 'pathogen burden', or the aggregate pathogen load, is a more significant risk factor than any single infection. In this respect, Prasad et al. (27) suggested that the presence of multiple intracellular pathogens in endothelial cells and the resulting endothelial dysfunction may be a mechanism contributing to the initiation of atherosclerosis and increased progression of established disease (28).

A possible mechanism by which infection may initiate and facilitate the progression of atherosclerosis may be explained in terms of the immune response to bacterial heat shock proteins (HSPs). All cells, both prokaryotic and eukaryotic, express HSPs on exposure to various forms of stress, including temperature, oxidative injury and infection (19, 26). Factors such as bacterial lipopolysaccharide, cytokines, and mechanical stress can induce the expression of host protective hHSP60 on endothelial cells. Due to the homologous nature of HSPs among species, there may be a cross-reaction of the immune response to the HSPs of the pathogens with the HSPs expressed by stressed endothelial cells. It has been postulated that cross-reactivity of antibodies to bacterial HSP (GroEL) with hHSP60 on endothelial cells may result in endothelial dysfunction and the subsequent development of atherosclerosis (35). A correlation between high anti-HSP60/65 antibody titers and high morbidity and mortality due to atherosclerosis has been demonstrated (21) and these antibodies were shown to be cross-reactive with those of other bacteria and were able to lyse stressed but not unstressed endothelial cells (20).

The aim of this study was to investigate the cross-reactivity of bacterial GroEL antibodies with hHSP60 in a group of patients with atherosclerosis and to quantify the presence of a panel of six microorganisms, including three bacteria implicated in periodontal disease, in atherosclerotic lesions.

Material and methods

Patients

Thirty-seven patients undergoing carotid endarterectomy at the Royal Brisbane and Women's Hospital were involved in this study. Peripheral blood was obtained from 22 of these patients and biopsy material from atherosclerotic plaques was collected from 25 patients from this group. Informed consent to use tissue that would otherwise have been discarded was obtained from each patient at the time of surgery. A written explanation of the purpose of the study was provided, and signed consent according to the Helsinki Declaration was obtained. Institutional ethics review committee approval to carry out the study was also obtained.

Data on current general medical conditions was obtained for 22 of the patients and periodontal status information for 18 of the atherosclerosis patients. A number of patients had hypertension (15/22) and hypercholesterolemia (12/22). Eight of the 22 patients had diabetes and four were current smokers. The age range of the patients was 55–84 years. Periodontal disease status was classified by a senior experienced periodontist as either healthy (no sites with pocket depth ≥ 3.5 mm), moderate (≥ 1 sites with pocket depth ≥ 3.5 mm and < 4 sites ≥ 5 mm) or advanced (≥ 4 sites with pocket depth ≥ 5 mm). Five of the 18 patients who consented to an oral examination were characterized as healthy, six had advanced periodontal disease and seven were edentate.

ELISA

Plasma was taken from the peripheral blood samples after Ficoll-Paque density centrifugation and peripheral blood mononuclear cells were collected for use in another study. Levels of anti-GroEL, anti-hHSP60 and anti-*P. gingivalis* IgG antibodies were measured using an ELISA technique described by Gemmell et al. (10, 11). Briefly, recombinant (r) GroEL (purified from *Escherichia coli*, Stressgen Biotechnologies Corporation, Victoria, Canada), rhHSP60 protein (does not contain GroEL as confirmed by the manufacturer, Stressgen) (2 μg/ml) or *P. gingivalis* ATCC 33277 (2) (1 μg/ml) were coated onto 96-well high-binding plates (Maxisorb Immunoplates, Nunc, Roskilde, Denmark). To block possible lipopolysaccharide in the preparations, 10 μg/ml polymyxin B in phosphate-buffered saline (PBS)-Tween 20 (0.05%) was added to the plates. Plasma samples diluted with PBS were used to determine baseline antibody levels. Plasma samples were also incubated with 10 μg/ml hHSP60 (determined from a pilot study) to absorb any hHSP60 antibody activity before being added to the plate. Peroxidase-conjugated rabbit antihuman IgG (DAKO, Glostrup, Denmark) was added, followed by detection with substrate containing 0.0075% H_2O_2 and 2.5 mM o-tolidine (Eastman Kodak, Rochester, NY). The optical density of the wells was read at an absorbance of 450 and 655 nm on a BIO-RAD Microplate reader Model 3550 (Philadelphia, PA). IgG antibody levels in the plasma samples diluted with PBS and those absorbed with hHSP60 were determined after correcting for the dilution of plasma samples with RPMI in Ficoll-Paque centrifugation, from a standard curve of dilutions of a known concentration of normal human IgG (Zymed, San Francisco, CA), which were coated onto each plate as described by Gemmell et al. (10, 11). Anti-*P. gingivalis* and anti-GroEL antibody levels were compared with and without absorption with hHSP60. As a positive control, anti-hHSP60 antibody levels were also measured with and without absorption with hHSP60. Negative control wells used PBS in place of the serum samples and were used to determine background values.

Polymerase chain reaction (PCR)

Endarterectomy specimens were quenched and stored in liquid nitrogen. From each of three different sites (approximately 1 cm apart) on the samples, 25 mg of tissue was minced, pushed through a 70 μm cell strainer (Falcon, Franklin Lakes, NJ) and DNA extracted using the NucleoSpin Tissue kit (Machery-Nagel, Easton, PA) according to the manufacturer's instructions. The purified DNA from each individual sample was eluted in a volume of 100 μl.

Detection of microorganisms using a ubiquitous bacterial marker

The presence of bacterial DNA in the samples was assessed by PCR using a ubiquitous primer that matches almost all bacterial 16S rRNA genes as described by Ashimoto et al. (1). Briefly, the DNA was amplified in 50 μl volumes containing 1.5 μM of primer (Proligo, Lismore, Australia), 1.5 mM $MgCl_2$, 200 μM nucleotides, standard PCR buffer, 1.0 U of AmpliTaq polymerase (Gibco-BRL/Life Technologies, Mount Waverley, Australia) and 5 μl of the extracted DNA. The PCR protocol consisted of a predenaturation step of 95°C for 2 min, followed by 36 amplification cycles (94°C 30 s; 55°C 1 min; 72°C 2 min), and a final step of 72°C for 10 min. The amplification reaction products were analyzed by gel electrophoresis and visualized by UV fluorescence.

Real time PCR to detect specific bacteria

A real time PCR system was used to determine bacterial numbers in the artery samples. Specific oligonucleotide sequences were derived from the 16S rRNA genes for *P. gingivalis*, *T. forsythia*, *Fusobacterium nucleatum, H. pylori, Chlamydia pneumoniae* and *Haemophilus influenzae* (GenBank, http://www.ncbi.nlm.nih.gov/GenBank/index.html). PrimerQuest (Integrated DNA Technologies Inc., Coralville, IA) was used to design the TaqMan probe and the forward and reverse PCR primer sets (Table 1). Probes and primers were initially assessed for species specificity by a BLAST (National Center for Biotechnology Information) search to determine homology to known sequences and later by attempting to amplify DNA from nontarget organisms (*Porphyromonas endodontalis* ATCC 35406, *Actinomyces viscosus* FDC 14, *Campylobacter rectus* FDC 371, *Capnocytophaga gingivalis* ATCC 33624, *Capnocytophaga sputigena* ATCC 33612, *Capnocytophaga ochracea* ATCC 33596, *Streptococcus sanguis, Streptococcus mutans* ATCC 10449, *Actinobacillus actinomycetemcomitans* FDC Y4, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 25261).

PCR amplification reaction mixtures (25 μl) contained the DNA sample (5 μl), a proprietary master mix (containing reaction buffer, nucleotides and Thermostat DNA polymerase (Abgene, Surrey, UK), fluorogenic probe (5 μM), primers (18 μM each) and reference dye (ROX 500 nM)). Reactions were performed in capped 0.2-ml thin-walled 96-well reaction plates (Abgene). The PCR protocol consisted of an initial step for enzyme activation (95°C for 15 min) followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were performed with the ABI Prism 7700 sequence detection system, and data were analyzed with the sequence detection system software (v1.9) (PE Applied Biosystems, Melbourne, Australia). Each real time PCR assay consisted of seven standards of known bacterial number and negative controls including DNA from nontarget species, in addition to the patient samples. The bacteria used for the standards was grown in this laboratory, apart from *C. pneumoniae*, which was provided by Professor Timms from the Queensland University of Technology, Australia. The amount of bacterial DNA as well as the number of bacteria in each sample was determined by comparing the amplification curve of each sample to those of the standards. The linearity and detection limits of the assays were determined by amplifying a series of 10-fold dilutions of the standards so that the correlation coefficient could be calculated from the standard curve of Ct values.

Statistics

Multivariate analysis of variance using the general linear model was used to test for differences in the antibody levels of the different periodontal status groups and of the patients grouped according to medical conditions. Pairs of groups were then tested for significance using Student's *t*-test. The Minitab statistical package (Minitab Inc., State College, PA) was used to perform the analyses. Comparisons of the prevalence of different species of microorganisms were made using the chi-squared test, based on the assumption that the presence of each bacteria was an independent event.

Table 1. Species-specific and ubiquitous primers and probes for PCR and real time PCR

Primer pairs (5'-3')	Probe (5'-3')
P. gingivalis	(6-Fam)AACGAGCGCAACCCACATCGGT (Tamra)
GGTGTCGGCTTAAGTGCCA	
CCTCAGCGAAAACTGTTAGCAA	
F. nucleatum	(6-Fam)TCGACGCAACGCGAGGAACCTT (Tamra)
CGGTGGAGCATGTGGTTTAA	
TTCCTAAGATGTCAAACGCTGG	
T. forsythia	(6-Fam)CCCATCCGCAACCAATAAA(Tamra)
GAAAGTCGGACTAATACCTCATAAAACAG	
CGTTACCTCACCAACTACCTAATGG	
H. pylori	(6-Fam)AAACAGGTGCTGCACGGCTGT CGTCAGCT(Tamra)
AGTGTCTGGCTTGCTAGACCTTGA	
TGCGGGACTTAACCCAACATCTCA	
H. influenzae	(6-Fam)AACTGGCGTAGTTGCAGGTTGC AGCAGCCA(Tamra)
ACCGCACTCTTAAGGAGAGATACT	
TCTGGTAAATAACCGCTAGCACCACG	
C. pneumoniae	(6-Fam)AGAGAGGCGCCCAAGGTGAGG CTGATGACT(Tamra)
CCGTCACATCATGGGAGTTGGTTT	
GCCCAACCTAGTCGGGTTGTTAAA	
Ubiquitous primer	
GATTAGATACCCTGGTAGTCCAC	
CCCGGGAACGTATTCACCG	

Results

ELISA

The 22 atherosclerosis patients were divided into four groups according to their periodontal status (Fig. 1). Analysis of plasma samples incubated with PBS showed that the mean levels of antibody to hHSP60, GroEL and *P. gingivalis* tended to be lower in the healthy group compared with the other periodontal status groups; however, this difference was not significant. The mean levels of anti-*P. gingivalis* antibodies were higher in those patients with advanced periodontal disease (434.0 ng/ml) than in the healthy patients (185.9 ng/ml) ($P < 0.014$). When the results of the group of 22 patients were analyzed according to general medical conditions there was only one significant association. Anti-*P. gingivalis* antibody levels were elevated in smokers (506.9 ng/ml) compared with nonsmokers (282.1 ng/ml) ($P < 0.039$).

When plasma samples were incubated with hHSP60, the levels of both anti-GroEL and anti-*P. gingivalis* antibodies were reduced compared with samples incubated with PBS, indicating a cross-reactivity with hHSP60. This was determined by the percent reduction of specific antibody titers for plasma samples incubated with hHSP60 compared with plasma samples incubated with PBS for each patient. There were large variations among individuals in the magnitude of the reduction of anti-GroEL and anti-*P. gingivalis* antibody levels following absorption with hHSP60, including some patients, particularly in the advanced periodontitis and healthy groups, who showed no evidence of cross-reactivity. The mean percent reduction of specific antibody titers tended to be lower in the periodontal health group than in the other groups; however, this was not significant (Table 2).

PCR

Detection of microorganisms using a ubiquitous bacterial marker

Bacterial DNA was detected in all 25 atherosclerotic arteries and in all but three of the 75 sites examined.

Real time PCR to detect specific bacteria

Using 10-fold serial dilutions of *P. gingivalis*, *F. nucleatum*, *T. forsythia*, *C. pneu-*

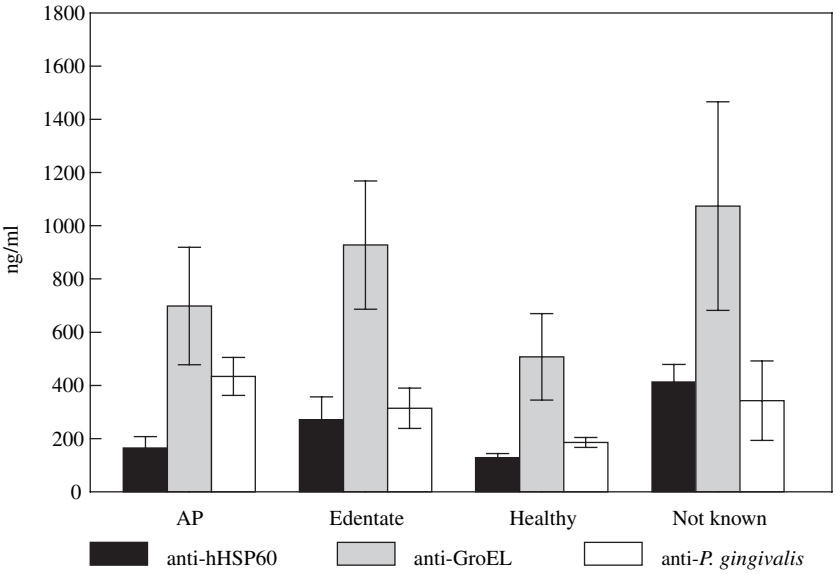

Fig. 1. Mean levels of anti-hHSP60, anti-GroEL, and anti-*P. gingivalis* antibodies (ng/ml) in plasma samples of 22 patients with atherosclerosis grouped according to periodontal disease status (advanced periodontal disease (AP) $n = 6$; edentate $n = 7$; healthy $n = 5$; not known $n = 4$).

moniae, *H. influenzae,* and *H. pylori* it was possible to detect DNA over a linear range from $1°–10^8$, $10^1–10^8$, $10^1–10^8$, $10^1–10^8$, $10^3–10^8$ and $10^3–10^8$ with corresponding Ct values from 16.2–45, 15–45, 18–45, 18–45, 33–45 and 24–45, respectively. The correlation coefficient of the threshold cycle plotted against the $\log_{10}$ of the dilution of *P. gingivalis*, *F. nucleatum*, *T. forsythia*, *C. pneumoniae*, *H. influenzae*, and *H. pylori* standards was 0.952, 0.997, 0.979, 0.967, 0.993, and 0.988, respectively. The numbers and species of microorganisms detected in DNA extracted from 25 mg of tissue from each of three sites of 25 atherosclerotic arteries are shown in Table 3. The median numbers of microorganisms detected from positive sites was much lower for *P. gingivalis* than for the other pathogens examined. However, *P. gingivalis* was shown to be present in all artery specimens (25/25), significantly higher frequency than that of any of the other microorganisms studied ($P < 0.001$): *F. nucleatum*, *T. forsythia*, *C. pneumoniae*, *H. influenzae*, and *H. pylori* were found in 84% (21/25), 48% (12/25), 28% (7/25), 4% (1/25), and 4% (1/25), respectively (Fig. 2). Multiple infections were observed to occur more commonly than single infections. *P. gingivalis* and *F. nucleatum* were present in 84% (21/25) of arteries and 40% (10/25) were positive for all three of the periodontopathogens, *P. gingivalis*, *F. nucleatum*, and *T. forsythia.* These organisms were present in a significantly greater number of arteries than were the remaining three microorganisms ($P < 0.05$). Three or more microorganisms were detected in 60% of arteries and 12% were positive for four or more of the panel examined.

Discussion

The results of the present study showed that levels of anti-*P. gingivalis* antibodies were significantly higher in atherosclerosis patients with advanced periodontitis compared with those with periodontal health. Those atherosclerosis patients who were smokers also had elevated levels of anti-*P. gingivalis* antibodies. This is in agreement with other studies that have demonstrated that elevated levels of serum antibodies to *P. gingivalis* are associated with periodontal disease (17) and smoking (4, 9). We have demonstrated that patients with periodontal health showed a trend towards lower levels of antibodies to hHSP60, GroEL, and *P. gingivalis*. Another report also showed that antibody levels to *P. gingivalis* GroEL and hHSP60 were significantly lower in healthy than in periodontal disease patients (36) and increased levels of salivary IgA anti-HSP65 antibodies have been observed in gingivitis patients (29). In contrast, Buhlin et al. (3) showed no differences in the levels of IgG anti-HSP60 and anti-HSP65 antibodies between patients with and those without periodontitis and reduced levels of IgA anti-HSP60 antibodies in periodontitis patients.

The results of the present study demonstrated the cross-reactivity of plasma anti-GroEL and anti-*P. gingivalis* antibodies with hHSP60 in patients with atherosclerosis. This cross-reactivity did not occur to the same degree in all individuals. Indeed, in some patients the antibodies to either or both GroEL and *P. gingivalis* showed no cross-reactivity with hHSP60. Although it did not reach significance, the mean reductions in specific antibody levels after incubation with hHSP60 tended to be lower in the healthy group compared with the other periodontal status groups. Yamazaki et al. demonstrated that antibody levels to *P. gingivalis* GroEL and hHSP60 were higher in periodontal disease patients who also had atherosclerosis than in patients with periodontal disease alone (36). Therefore those patients with atherosclerosis and periodontal disease may represent a group with increased respon-

Table 2. Percentage reduction of specific antibody titres after absorption with 10μg/ml hHSP60

AP* (n=6)			Edentate (n=7)			Healthy (n=5)			Not known (n=4)		
Patient No.	anti-GroEL	anti-*P.g*†	Patient No.	anti-GroEL	anti-*P.g*†	Patient No.	anti-GroEL	anti-*P.g*†	Patient No.	anti-GroEL	anti-*P.g*†
1	36.1	20.1	5	12.5	18.8	9	8.9	11.8	2	49.1	no data
3	0.0	27.9	6	19.0	29.3	12	0.0	0.0	4	3.6	25.3
13	0.0	10.5	8	0.0	12.0	14	7.1	0.0	7	5.1	4.0
15	11.7	0.0	10	4.8	2.3	17	0.0	3.1	20	0.0	16.7
18	0.0	0.0	11	57.9	13.4	21	6.5	25.1			
19	0.0	0.0	16	32.4	0.0						
			22	14.6	29.5						
Mean	8.0	9.7		21.1	12.6		4.5	8.0		14.5	15.3
SE	5.9	4.9		8.7	4.4		1.7	4.4		9.5	4.4

†Anti-*P. gingivalis*.
*Advanced periodontal disease.

Table 3. Numbers of microorganisms detected per 25 mg of tissue from each of three sites of endarterectomy specimens from 25 patients (median no. of microorganisms determined from all positive sites)

	P. gingivalis			*F. nucleatum*			*T. forsythia*			*H. influenzae*			*H. pylori*			*C. pneumoniae*		
Patient No.	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
2	2	2	7	1.1×10^3	570	370	4.1×10^3	0	0	0	0	0	0	0	0	0	0	0
3	9	0	0	990	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3×10^6
5	2	0	32	2×10^3	840	0	0	0	0	0	0	0	0	0	0	0	0	0
6	2	0	0	320	410	0	0	0	0	0	0	0	0	0	0	0	0	0
7	5	98	0	0	0	0	0	0	4.9×10^6	0	0	1.1×10^6	0	0	6.6×10^3	0	5.7×10^4	1.2×10^5
8	0	41	43	0	650	390	0	6.5×10^9	0	0	0	0	0	0	0	0	0	0
9	27	0	0	3.2×10^3	0	0	8.8×10^3	0	0	0	0	0	0	0	0	0	0	0
20	6	0	5	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	11	0	0	1.5×10^3	0	0	2.7×10^3	0	0	0	0	0	0	0	0	0
22	56	7	0	3.7×10^3	0	5×10^9	2.9×10^6	1.2×10^5	0	0	0	0	0	0	0	0	0	4.8×10^4
23	21	0	0	1.4×10^3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	17	0	1	64	0	0	0	0	0	0	0	0	0	0	0	0	6×10^4	4.3×10^5
25	2	0	0	840	530	0	1.2×10^4	0	0	0	0	0	0	0	0	0	0	
26	4	17	15	3×10^3	290	2.1×10^3	2.8×10^4	0	0	0	0	0	0	0	0	0	0	0
27	0	14	0	140	740	0	0	0	0	0	0	0	0	0	0	0	0	0
28	460	0	0	2.7×10^4	31	96	1.2×10^5	0	0	0	0	0	0	0	0	0	0	6.6×10^4
29	6	4	3	370	2.9×10^3	240	2.6×10^3	1.5×10^4	0	0	0	0	0	0	0	0	0	0
30	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	2	1	0	0	0	0	1.3×10^4	0	0	0	0	0	0	0	0	0	0	0
32	0	26	25	320	0	8.7×10^8	0	0	0	0	0	0	0	0	0	0	0	5.4×10^4
33	6	0	6	0	0	1.3×10^3	0	0	0	0	0	0	0	0	0	0	0	0
34	0	29	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	14	4	0	1.2×10^3	0	2.1×10^8	0	0	0	0	0	0	0	0	0	0	0	2.3×10^6
36	28	0	0	1.6×10^3	0	93	0	0	0	0	0	0	0	0	0	0	0	0
43	0	3	4	1.6×10^3	0	530	2.1×10^3	0	0	0	0	0	0	0	0	0	0	0
Median		7			840			1.4×10^4			1.1×10^6			6.6×10^3			6.6×10^4	

siveness and cross-reactivity to HSPs. The variation observed within the periodontal status groups in the degree of cross-reactivity of antibodies to GroEL and *P. gingivalis* with hHSP60 in the present study would suggest that cross-reactivity of the immune response to HSPs may be important as a possible mechanism in atherogenesis for some individuals, but not others.

The finding of bacterial DNA in all of the artery biopsies supports the involvement of infectious agents in atherosclerosis. Unlike previous studies, the present study utilized real time PCR to quantify the panel of three periodontopathic bacteria and three systemic pathogens. *P. gingivalis* was found in all specimens, albeit at very low numbers, in contrast to two other studies that have reported much lower detection rates of 15–26% (13, 31). A recent study found that of the patients whose subgingival plaque tested positive for periodontopathogens using PCR, 85% were positive for *P. gingivalis* and 16% for *T. forsythia* in the artery walls of abdominal aortic aneurysm biopsies (15). In this study, most of the *P. gingivalis*-positive patients had severe periodontal disease. However, in the present study the classification of periodontal disease was much stricter, resulting in more patients being classified as periodontally healthy and fewer with severe periodontal disease. In addition, our higher detection rates of these organisms may be due to the increased sensitivity of the real time PCR technique, which has been reported to be a more reliable indicator of the presence of *C. pneumoniae* than cell culture and nested PCR (33), as well as the fact that Kurihara et al. (15) only tested one site per lesion. The distribution of the microorganisms within the artery was not homogeneous, demonstrating that the analysis of multiple sites within a specimen may increase the sensitivity of the detection assay. *T. forsythia* has previously been reported to occur in 30% of carotid endarterectomy specimens (13), which is lower than our findings. The reported detection rates of *C. pneumoniae* in atherosclerotic tissue vary from 0% (25) to 79% (22), possibly due to the use of different techniques.

The low numbers of *P. gingivalis* demonstrated in the atherosclerotic lesions would suggest that this organism is unlikely to play a role at the site of the lesion and is present as a consequence of the lesion. The bacteria may have passively accumulated here from the circulation as a result of the presence of the lesion. The presence of this organism,

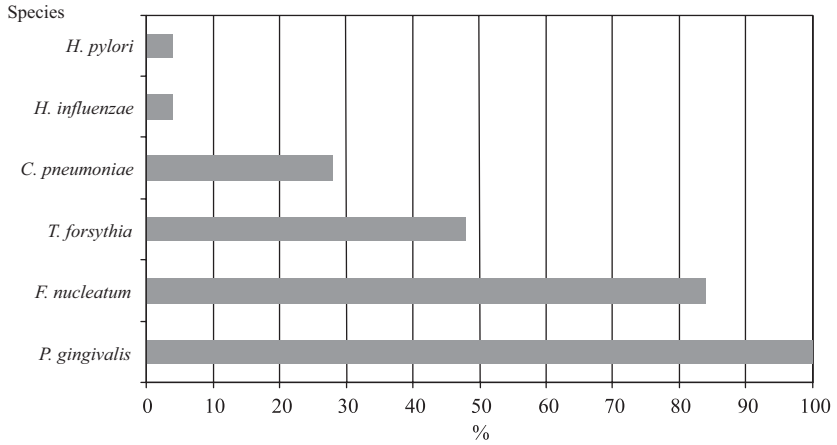

Fig.2. Prevalence of microorganisms detected by real time PCR in atherosclerotic lesions of 25 patients.

however, in all of the specimens examined, suggests that it has colonized the oral cavity and caused infection in all of these atherosclerosis patients at some point and, for the 10 patients for whom data were available, this was regardless of their current periodontal status. This was also supported by our findings of measurable levels of anti-*P. gingivalis* plasma antibodies for these 10 patients. The prevalence of periodontopathic bacteria significantly outweighed that of the other microorganisms examined in this study, suggesting that infection with these organisms may occur more commonly than infection with the remaining three microorganisms studied. Apart from *P. gingivalis*, the higher numbers of the microorganisms detected in this study may indicate a possible local role for these in the progression of the lesion. However, it is possible that remote sites of infection are more important. The hypothesis that the cross-reactivity of the immune response to bacterial HSPs with hHSP60 expressed by stressed endothelial cells would mean that *P. gingivalis* may not need to be present within the lesion to stimulate a specific humoral and cellular response which may cause initiation and/or progression of the atherosclerotic lesion. Since this study has shown that periodontopathic bacteria may have caused infection more commonly than the other organisms traditionally associated with atherosclerosis, it is likely that periodontopathic bacteria contributed a significant proportion of the infectious burden for each of the atherosclerosis patients in the present study. The concept that aggregate pathogen load may be a significant risk factor for atherosclerosis is strengthened by our findings that multiple pathogens were present in the majority of arteries examined.

In conclusion, we have demonstrated cross-reactivity of plasma anti-GroEL and anti-*P. gingivalis* antibodies with hHSP60 in a number of atherosclerosis patients, and a highly variable degree of cross-reactivity. We found bacterial DNA in all 25 arteries examined. The prevalence of the three periodontopathic bacteria was shown to be significantly higher than that of the other three microorganisms. These results lend support to the hypothesis that in some patients, the cross-reactivity of the immune response to bacterial HSPs, including those of periodontal pathogens, with arterial cells expressing hHSP60 may be a possible mechanism for the apparent association between atherosclerosis and periodontal infection. Should infection be established as a risk factor for atherosclerosis, then effective treatment of chronic infections such as periodontitis would have an important preventive role in atherosclerosis. It is likely, however, that many factors are involved in this disease process and that the infectious component may be more significant for some patients than for others.

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