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Review and Hypothesis

Involvement of periodontopathic biofilm in vascular diseases

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Oral bacteria inhabit biofilms, which are firm clusters adhering in layers to surfaces and are not easily eliminated by immune responses and are resistant to antimicrobial agents. Dental plaque is one such biofilm. In the past 10 years, subgingival plaque bacteria forming biofilms have been increasingly reported to be involved in systemic diseases. A close relationship between microbial infections and vascular disease has also been reported in the past two decades. The present review discusses the significance of the ecologic characteristics of biofilms formed by periodontopathic bacteria in order to further clarify the associations between periodontal disease and systemic disease. We focus on the relationships between periodontal disease-associated bacteria forming biofilms and vascular diseases including atherosclerosis and carotid coronary stenotic artery disease, and we discuss the direct and indirect effects on vascular diseases of lipopolysaccharides as well as heat shock proteins produced by periodontopathic bacteria.
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Periodontopathic biofilms in periodontal pockets

Bacteria in nature often occur as biofilms, which are firm clusters of bacteria adhering in layers to some kind of substrate, not as floating planktonic cells. Such biofilms are not easily eliminated by an immune response and are resistant to antimicrobial agents (Costerton *et al*, 1987; Costerton, Stewart and Greenberg *et al*, 1999). Therefore, in a sense, researchers have generally investigated bacteria in the wrong state. In environments in which bacteria normally live and grow, they have the means of signaling and receptors for such signals; thus they can communicate with each other. When the environment deteriorates, they emit specific signals and stop growing (Whitehead *et al*, 2001). The signals that transmit such information may be called bacterial pheromones or hormones. Because they induce their own phenotypes, they are called autoinducers (Greenberg, 1997). Many factors respond to autoinduction, including the passage of genes, motility for seeking nutrients, growth, survival, and toxin production. The glycocalyx of many bacterial species, including those of periodontopathic bacteria, play significant roles, not only in forming biofilms, but also in escaping from host defense mechanisms such as phagocytosis and killing by leukocytes.

Dental plaque is a unique ecosystem. Several hundred bacterial species inhabit the human oral cavity (Tanner *et al*, 1998), and these multiple bacterial species form a community as dental plaque (Kolenbrander, Anderson and Moore, 1989; Kolenbrander, 2000). Bacteria in periodontal pockets use gingival crevicular fluid as the nutrient source of carbon and nitrogen, as well as of essential growth factors such as minerals and vitamins. These bacteria then proliferate and communicate by signals to each other (Carlsson, 2000; Palmer *et al*, 2001). In order to maintain the ecosystem, various anaerobes anchor to each other by forming aggregated bacterial masses, as shown in Figure 1 (Kigure *et al*, 1995). The regulation of bacterial gene expression in response to changes in cell density is known as quorum sensing. Quorum-sensing bacteria synthesize and secrete extracellular signaling molecules called autoinducers, which accumulate in the environment as the population increases. Recent studies have demonstrated that periodontopathic bacteria produce these extracellular signals (Chung *et al*, 2001; Fong *et al*, 2001; Frias, Olle and Alsina, 2001). These studies have also indicated that the signals produced by subgingival bacteria induce both intra- and inter-species responses in the mixed-species microbial communities that exist in the oral cavity.

Natural and acquired immune responses play significant roles in the elimination of invading microorganisms.

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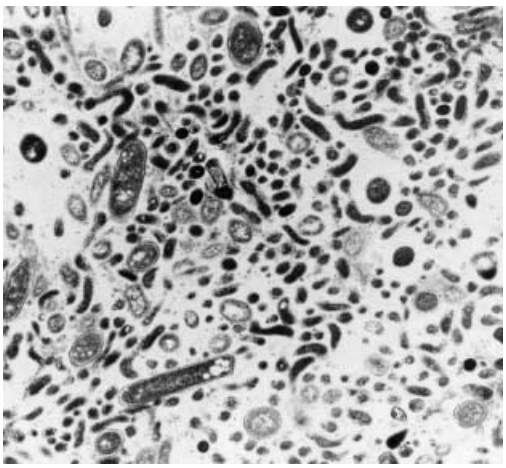

Figure 1 Electron micrograph of an ultrathin section of periodontopathic biofilm in a periodontal pocket. Note the aggregated bacterial mass with predominant gram-negative bacteria including Treponemes

T cells and B cells show prompt responses against protein antigens. However, T cells cannot respond to polysaccharide antigens such as glycocalyx, which is a biofilm matrix. Therefore, host defense immunologic responses do not appropriately occur against biofilm matrix antigens. Even if a specific antibody that can function to eliminate biofilm is produced, it acts only on the surface of bacterial cells in dental plaque. Moreover, although the immunologic response related to phagocytosis is able to eliminate bacteria on the surface of biofilms, phagocytic cells cannot phagocytose or kill the bacterial cells within the biofilm. Although disinfectants and antibiotics are effective against floating bacteria, these agents cannot penetrate as deeply as the central bacterial cells in biofilm. In addition, because bacteria in the central region are in the stationary phase, bacteriostatic antibiotics inhibiting metabolism are not effective. These evading factors of dental plaque, a well-studied biofilm, result in the persisting infection in subgingival regions.

Dental plaque bacteria can enter the blood stream

It has been repeatedly reported that dental plaque bacteria forming biofilms can enter the bloodstream, resulting in bacteremia. The risk of bacterial endocarditis increases as the oral hygiene index and the bacterial count increase (Strom *et al*, 2000). *Streptococcus sanguinis* ('sanguinis' means 'of blood') is often isolated from the peripheral blood. *Streptococcus sanguinis* forms biofilms and is the *Streptococcus* species most commonly found in dental plaque, indicating that dental plaque biofilm is one source of bacteria leading to bacterial endocarditis (Larsen and Fiehn, 1996; Chayakul *et al*, 2002). Periodontopathic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* have also been found in patients with endocarditis (Van Winkelhoff and Slots, 1999). Recently, it has been shown that periodontal inflammation with periodontitis enhances bacteremia (Andersen *et al*, 2003, *J Dent Res Special Issue B*, Abstract 1159). These findings indicate that dental plaque bacteria can enter the blood stream.

Ford *et al* (1993), Herzberg *et al* (1997), Herzberg and Weyer (1998), and Meyer, Cong and Herzberg (1998) have all demonstrated the platelet clotting activity of *S. sanguinis*. We also demonstrated that the lipopolysaccharides (LPSs) of periodontal disease-associated bacteria can hemagglutinate human blood (Okuda and Kato, 1987). *Porphyromonas gingivalis* also possesses platelet aggregation activity (Sharma *et al*, 2000). These findings indicate that oral biofilm-forming bacteria that enter the bloodstream can affect the circulation by clotting blood components.

Detection of periodontopathic bacteria in samples of stenotic artery plaques and atherosclerotic lesions

Circulatory disorders such as atherosclerosis involve very complex interactions between factors such as hyperlipidemia, obesity, smoking, and genes. It has been clarified in recent years that *Chlamydia pneumoniae*, which is related to respiratory disease, enters the circulation, invades the vascular endothelial cells, and triggers atherosclerosis at the invasion site (Daus *et al*, 1998; Ross, 1999; Parchure, Zouridakis and Kaski, 2002). Human cytomegalovirus has also been demonstrated to be a factor leading to atherosclerosis (Daus *et al*, 1998). In consequence, atherosclerosis has been considered to include a microbial infection-induced inflammatory disorder.

Some epidemiologic studies have shown a relationship between periodontal disease and ischemic arterial disease (Beck *et al*, 1996, 1999; Beck and Offenbacher, 2001). In contrast, another study (Hujoel *et al*, 2000), found no epidemiologic relationship between periodontal disease and heart disease. Many researchers have already reviewed the relationship between oral bacteria and vascular diseases (Herzberg *et al*, 1997; Herzberg and Weyer, 1998; Okuda and Ebihara, 1998; Paquette *et al*, 1999; Pallasch and Slots, 2000; Slavkin and Baum, 2000; Kuramitsu *et al*, 2001; Slots and Kamma, 2001; Genco, Offenbacher and Beck, 2002; Kuramitsu, 2002).

Postulated mechanisms that may link periodontal disease-associated biofilms and vascular disease are shown in Figure 2. Bacterial species in periodontal pockets have been detected in atherosclerosis and samples from the lesions (Chiu, 1999; Haraszthy *et al*, 2000). We have also detected periodontopathic *Treponema denticola* in samples of atherosclerosis lesions (Okuda *et al*, 2001). We extracted DNA from embedded arterial disease samples and searched for *T. denticola* 16S rRNA in six aneurysm lesions. When the samples were stained with a specific anti-*T. denticola* antibody, we observed aggregated antigenic particles reacting with the antibody in and around the foam cells in aneurysms (Figure 3). All the samples which were PCR- and immunofluorescent-positive were thin sections of aneurysms. However, we could not detect any DNA or

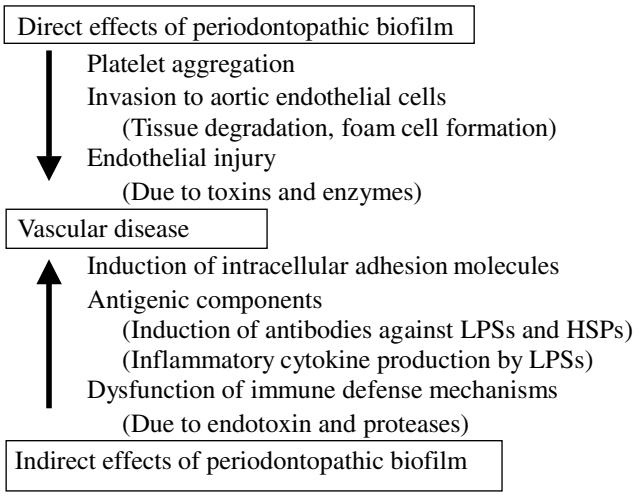

Figure 2 Postulated mechanisms that link the periodontopathic biofilm with vascular disease

antigens of *T. denticola* in any of the 14 non-diseased aorta samples from deceased persons.

In cooperation with a heart surgery center, we extracted DNA from atheromatous plaque samples from coronary arterial walls at the sites of arteriosteno-sis and examined them for the presence of specific DNAs of periodontal disease-associated bacteria. We detected 16S rRNA of *P. gingivalis*, *A. actinomycetemcomitans*, *Campylobacter rectus*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), and *T. denticola* (Okuda *et al*, 2003, *J Dent Res* 82, Special Issue B, Abstract 0121). These findings are clear evidence that periodontal pocket bacteria enter the circulation.

Invasion of periodontopathic bacteria into epithelial cells

Using an antibiotic protection assay and electron microscopy, Dorn, Dunn and Progulske-Fox (1999) demonstrated that *P. gingivalis*, *Prevotella intermedia* and *Eikenella corrodens* invade coronary artery cells at a significant level. Katz *et al* (2000, 2002) showed that *P. gingivalis* induced degradation of epithelial cell junctional complexes and proteins. In addition, Schenkein *et al* (2000) and Rudney, Chen and Sedgewick (2001) showed that these periodontal disease-associated bacteria are able to invade human cells, including those of the vascular epithelium. Recently, we have demonstrated that cells of *T. denticola* can easily invade various epithelial cells, as shown in Figure 4 (Kimizuka, Ishihara, Kato and Okuda, unpublished data). Furthermore,

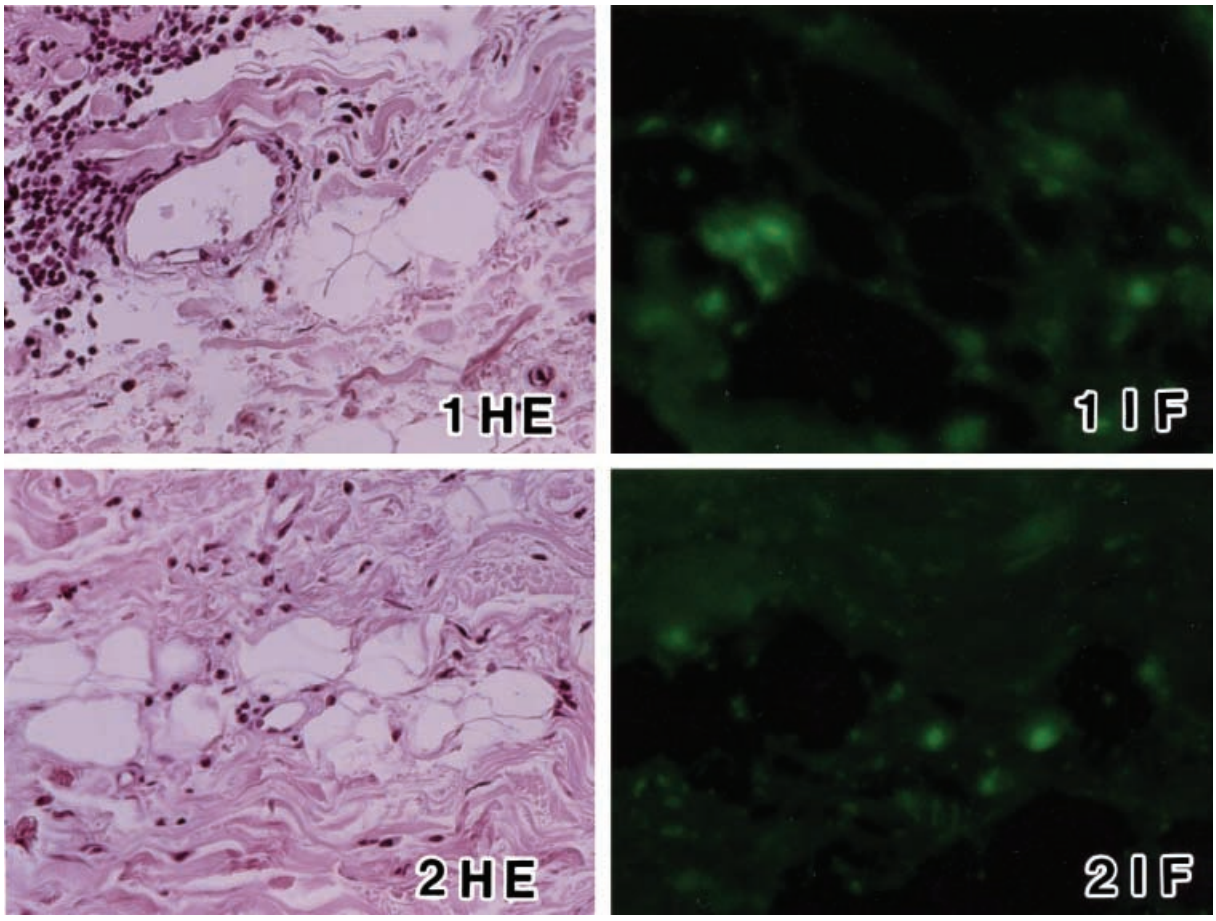

Figure 3 Hematoxylin–eosin staining of aortic aneurysm (1HE and 2HE). *Treponema denticola* antigens were detected around the foam cells (1IF and 2IF). *Treponema denticola*-specific DNA was detected in the DNA samples extracted from the two regions

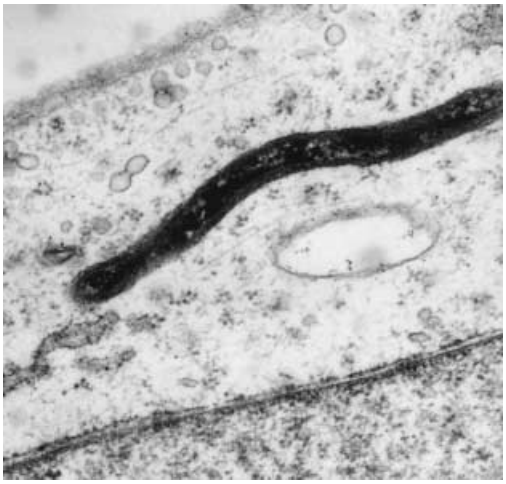

Figure 4 Electron micrograph of an ultrathin section of *Treponema denticola* invading an epithelial cell

when a heterozygous apolipoprotein E-deficient murine model, known as an atherosclerosis-accelerated model, was fed with a high-fat diet and inoculated with *P. gingivalis* cells, atherosclerosis was further promoted (Li *et al*, 2002). They concluded that *P. gingivalis* infection accelerated atherosclerosis in addition to the effects of the genes and environmental circumstances including the high lipid diet. Coronary arterial disease is a very frequent cause of death, and there are many patients with periodontitis. It is important to accumulate further reliable evidence concerning the relationship between periodontitis and cardiovascular diseases.

Role of periodontopathic bacterial lipopolysaccharides

Periodontopathic bacterial LPSs may have variable roles in systemic diseases. Wang and Ohura (2002) found that gingival fibroblasts express CD14, toll-like receptor (TLR) and binding of LPSs to CD14 and TLR on the cells activates various second-messenger systems. Recently, several research groups (Hajishengallis *et al*, 2002; Ogawa *et al*, 2002) have demonstrated that *P. gingivalis* LPS and lipid A induced cellular activation through TLR and subsequently induced tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β). These findings indicate that LPS and the components of periodontopathic bacteria can penetrate into the bloodstream through TLR on gingival fibroblasts. Rice *et al* (2003) found that clinically relevant levels of endotoxin have profound inflammatory effects on intact human saphenous veins. In addition, TLR polymorphism attenuates receptor signaling, diminishes the inflammatory response to gram-negative bacteria, and is associated with a decreased risk of atherosclerosis (Kiechl *et al*, 2002). These findings are all consistent with the hypothesis that innate immunity such as TLR function may play a part in atherogenesis.

Genetic background has been considered to be an important factor in colonization by bacteria. The genetic control of immune responses against periodontopathic bacteria has been studied by several groups (Watanabe, Marsh and Ivany, 1989; Shimauchi, Ogawa and Hamada, 1991; Honma *et al*, 1997). We investigated a variety of humoral immune responses to *A. actinomycetemcomitans* LPS and the production of cytokines in inbred strains of mice; our results suggest that there is a genetic control of responses to periodontopathic bacterial LPS (Kato *et al*, 2000a). It is well known that periodontopathic bacterial LPSs are able to induce several cytokines, including inflammatory ones, and possibly to affect the cytokine network. We demonstrated that the periodontopathic bacterial LPSs are cross-reactive with antibodies against cytokine molecules, human IL-1β, IL-4, IL-5, IL-6, IL-10, TNF-α, and interferon-γ (Kato and Okuda, 2001). In addition, the levels of IL-6 and IL-8 from human gingival fibroblasts treated with *P. gingivalis* LPS were found to be significantly higher than those from unstimulated control cells (Imatani, Kato and Okuda, 2001). These findings suggest that periodontopathic bacterial LPSs affect the cytokine network that plays a significant role to control homeostasis, results in the triggering of inflammatory responses.

Involvement of periodontopathic bacterial heat shock proteins

In most cells, heat shock proteins (HSPs) are present and serving as molecular chaperones; they play a role in cell protection from damage in response to stress stimuli. Bacterial cells, including periodontopathic bacteria and dental plaque samples, also produce HSPs, and many of these antigens cross-react with human HSPs. Recently, several groups have reported that soluble HSPs specifically bind to the TLR, initiating an innate immune response that includes the production of proinflammatory cytokines by macrophages and adhesion molecules in endothelial cells via NF-κB activation (Asea *et al*, 2002: Habich *et al*, 2002). Antibody responses against HSPs have been implicated in cardiovascular pathology (Kleindienst *et al*, 1995). The presence of elevated anti-HSP immunoglobulin G antibodies was shown to be independently associated with cardiovascular disease, supporting the hypothesis that cross-reactive HSP responses may contribute to disease progression (Mahdi *et al*, 2002). In addition, the titers of autoantibodies against HSPs are significantly elevated in patients with atherosclerosis, and responses to HSPs have been found in atherosclerotic plaques. Proinflammatory responses such as autoimmune reactions to HSPs in the vein wall can contribute to the initiation of atherosclerosis. Therefore, it is possible that HSPs have a general role in the response of the arterial wall to stress and may serve as a mediator/inducer of atherosclerosis in particular circumstances.

There is also growing evidence that the immune response is involved in atherosclerosis. Antibodies to HSPs have been shown to be a risk factor for carotid atherosclerosis (Xu, 2002). A significant association between the prevalence and severity of atherosclerosis in the carotid and femoral arteries and antibodies to

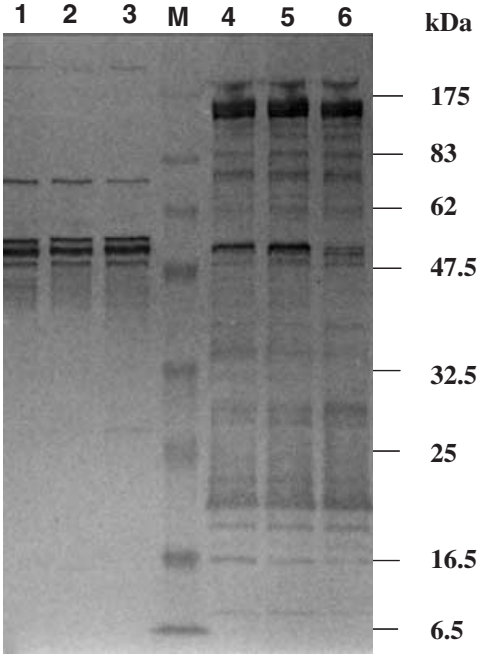

Figure 5 Reactivity of sonically extracted antigens from *Helicobacter pylori* and *Campylobacter rectus* strains with anti-*H. pylori* antibody. Lanes 1–3 show *H. pylori* strains including an ATCC strain; lanes 4–6, *C rectus* strains including ATCC strains; and lane M, the molecular size standard

C. pneumoniae has also been demonstrated (Birnie *et al*, 1998). Seropositivity to not only *C. pneumoniae* but also to *Helicobacter pylori* has been evaluated as a cardiovascular risk factor in a population-based study (Mayr *et al*, 2000). A recent study has shown that infections with *H. pylori* and *Chlamydia* spp. enhance production of antibodies to these pathogens may predispose to human atherosclerosis (Chemiela *et al*, 2003). In our study of the ecologic characteristics of *H. pylori* infections, we found that periodontopathic bacteria such as *Fusobacterium nucleatum* and *P. gingivalis* trap *H. pylori* cells but that oral bacteria also inhibit the growth of *H. pylori* in the human oral cavity (Ishihara *et al*, 1997; Okuda *et al*, 2000). Then it was found that *Campylobacter rectus* multiplying in chronic periodontitis lesions possess antigens including HSPs that cross-react with *H. pylori* (Ishihara *et al*, 2001). These cross-reactive antigens of *Campylobacter rectus* and *H. pylori* are shown in Figure 5. Furthermore, we found that salivary IgG and IgA titers against the two bacterial species were correlated, showing that the two bacteria induce cross-reactive immune responses to each other. It is very likely that such immune responses lead to inflammation by forming immunocomplexes; such cross-reacting antigens, including HSPs, may be linked to vascular disease.

A recent study also showed that the persistently elevated antibody levels against HSPs induced by chronic infection with these microorganisms are one of the risks of vascular diseases (Huittinen *et al*, 2003). Persistently, but not transiently, elevated antibodies against HSPs, especially when present together with an elevated C-reactive protein (CRP) level, predict

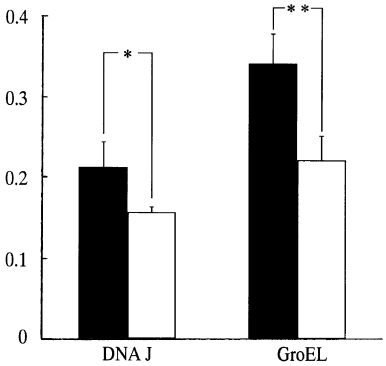

Figure 6 The average IgG titers obtained for DNAJ, which is the HSP of *Actinobacillus actinomycetemcomitans*, and Gro-EL, which is the HSP of *Escherichia coli*, in sera from patients with pustulosis palmaris et plantaris and from control healthy adults. There were significantly higher titers in the sera from patients with pustulosis palmaris et plantaris than in that from the control subjects. $*P < 0.05$, $**P < 0.01$

coronary events. We and other research groups have demonstrated that periodontopathic bacteria in persistent infections produce HSPs and that IgG responses to these HSPs were significantly higher in patients with periodontal disease than in the control group without periodontal disease (Ando *et al*, 1995; Sims *et al*, 2000; Yoshida *et al*, 2001). The serum IgG antibody titers against the HSPs of *Escherichia coli*, GroEL, and of *A. actinomycetemcomitans*, DNAJ, are shown in Figure 6 (Ishihara *et al*, 2000). In addition, we found that the antibody titers against *E. coli* GroEL significantly decreased after the treatment of the periodontal disease. Recently, it has been postulated that infections with virulent CagA-bearing *H. pylori* strains may contribute to the pathogenesis of early atherosclerosis by aggravating immune-inflammatory reactions (Mayr *et al*, 2003). It will be important to analyze the relationship between the antibody responses to periodontal disease-associated bacteria and vascular diseases in future studies.

Strategy for affecting the formation of periodontopathic biofilms

We would like to postulate three strategies for inhibiting the formation of periodontal bacterial biofilms. Periodontopathic bacteria that form biofilms are well known to be resistant to natural and acquired immune responses (Kolenbrander *et al*, 1989; Kolenbrander, 2000). Therefore, early colonization inhibition by periodontopathic bacteria by immunization may be a valid strategy. We have reported the induction of antibodies against colonizing factors of *P. gingivalis* and *A. actinomycetemcomitans* (Okuda *et al*, 1988; Harano, Yamanaka and Okuda, 1995). Attempts to induce protective immune responses against *P. gingivalis* have also used other pathogenic factors (Genco *et al*, 1998; Katz, Black and Michalek, 1999; Kawabata *et al*, 1999; Yonezawa, Ishihara and Okuda, 2001). Further studies are required to develop a safe and effective vaccine that can inhibit colonization by periodontopathic bacteria.

Interference against biofilm-forming signals is also a potential strategy. Finding inhibitory substances that affect biofilm formation in the periodontal region may help us to produce new drugs and drug delivery systems for elimination of periodontopathic bacteria.

Using salivary functions such as production of the innate antimicrobial component or specific secretory IgAs could also be effectual strategies against periodontopathic bacteria. The secretory IgA most abundant in saliva is the IgA type 1 subclass, which reacts with protein antigens, but the amount of IgA type 2 subclass, which reacts with polysaccharide antigens such as biofilm matrix, is low in the saliva. However, it has recently been demonstrated by many groups that the innate defense mechanisms of salivary proteins such as histatins and cystatins can plays significant role in prevention the colonization of bacterial species in the oral cavity (Imatani, Kato and Okuda, 2000; Kato *et al*, 2000b, 2002; Nieuw Amerongen and Veerman, 2002). It is also possible to use such synthesizing salivary components as histatins and cystatins to enfeeble the periodontopathic bacterial biofilm formation and/or their pathogenicities.

Further studies will clarify the importance of prevention and treatment of periodontal disease for health promotion. Attention should be focused on the new challenge of precise prevention of biofilm formation by periodontopathic bacteria.

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