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Effects of *Porphyromonas gingivalis* antigens and proinflammatory cytokines on human coronary artery endothelial cells

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Objective: Individuals with periodontitis have been cited as having a significantly increased risk of developing coronary heart disease. Although accumulating evidence suggests that periodontal infection is involved in the development and progression of atherosclerosis, the underlying mechanisms remain to be elucidated. In the present study, we examined how periodontal infection could contribute to endothelial dysfunction.
Methods: Human coronary arterial endothelial cells were stimulated with tumor necrosis factor (TNF)-α and interleukin (IL)-1β, both of which are reported to be elevated in the serum of periodontitis patients. Cells were also stimulated with lipopolysaccharide, outer membrane protein and heat shock protein 60 derived from *Porphyromonas gingivalis*, a representative periodontopathic bacterium which is known to stimulate myeloid cells.
Results: Although TNF-α and IL-1β, at concentrations a little higher than those in sera of periodontitis patients, up-regulated the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, *P. gingivalis* antigens had only a slight stimulatory effect.
Conclusion: Experiments in which the total pathogen burden is considered, rather than a single species of bacteria, would increase our understanding of the contribution of which periodontal infection to atherogenesis.

Key words: atherosclerosis; endothelial cell; periodontitis

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It has been suggested that periodontal infection may be involved in the initiation and progression of atherosclerosis and subsequent coronary heart disease. This is supported not only by epidemiologic case control studies (6, 24), but also by animal experiments using apolipoprotein E-deficient mice (16, 17).

In atherosclerosis it is recognized that both systemic and localized inflammation in the arteries contribute to the initiation and progression of the disease (28). A number of potential triggers capable of inducing proinflammatory cellular responses have been identified; these include modified lipoproteins, cytokines, chemokines, hypertension, smoking and diabetes mellitus (36). However, it has been hypothesized that one or more infectious agents may play a role in atherogenesis, either through a direct proinflammatory effect on the vessel wall or through a less specific, long-distance proinflammatory effect. In this context, it has been suggested that periodontal infection may be one such inflammatory foci.

Experimental evidence strongly implicates sustained elevated endothelial cell expression of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin, as mediators of leukocyte accumulation in atherosclerosis (28). In

addition to the expression of adhesion molecules, endothelial cells also produce chemokines and cytokines, further aggravating the inflammation. For example, monocyte chemoattractant protein-1 (MCP-1) appears responsible for the direct migration of monocytes into the intima at sites of lesion formation (9). Interleukin (IL)-6, which is produced by endothelial cells, has been implicated in the pathogenesis and clinical course of atherosclerotic vascular disease as it stimulates acute phase proteins and induces endothelial dysfunction (40). The expression of these adhesion molecules is regulated at least in part by circulating proinflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β and lipopolysaccharide derived from Gram-negative bacteria such as *Escherichia coli* (10).

Lipopolysaccharide is a potent activator of cells of the immune and inflammatory systems, including monocytes, macrophages, and endothelial cells. In addition it contributes to both local and systemic inflammation (35). It is now clear that responsiveness to lipopolysaccharide from *Porphyromonas gingivalis*, a major periodontopathic bacterium, can be conferred by Toll-like receptor 4 (TLR4) and TLR2 (5), both of which are expressed in human atherosclerotic lesions (7). Recent evidence showed that the Asp299Gly TLR4 polymorphism, which attenuates receptor signaling, is associated with a decreased risk of atherosclerosis, suggesting a pathogenic role for lipopolysaccharide (14).

As with other infectious agents, periodontal pathogens have been considered to be candidate triggers of the inflammatory response. Furthermore, it has been proposed that patients with periodontitis may have elevated circulating levels of some of these inflammatory markers (23). In addition, low levels of endotoxemia in apparently healthy subjects might result from chronic or recurrent infection associated with the breaching of epithelial barrier function such as seen in periodontitis (27).

Although it is reasonable to assume that periodontal infection stimulates not only cells in the inflamed periodontal tissue but also circulating immune cells to produce proinflammatory cytokines, it is as yet unknown how periodontopathic bacteria are involved in atherogenesis.

As periodontitis is caused by a number of periodontopathic bacteria, the examination of the role of a single bacterium may underestimate the effect of periodontal infection on atherogenesis but it will nevertheless allow us to make an assessment of the direct versus indirect effect of the organism.

Material and methods

Cell culture

Cryopreserved human coronary arterial endothelial cells (HCAECs) were purchased from BioWhittaker, Inc. (Walkersville, MD). The cells were grown in EBM-2 supplemented with EGM-2 MV (5% fetal bovine serum, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, ascorbic acid, human epidermal growth factor, gentamicin/amphotericin-1000, growth medium; BioWhittaker) and the growth medium was changed every other day until confluence. The cells between 8 and 12 passages were used. For stimulation, the cells were cultured in the growth medium at a concentration of 4×10^4 cells/well in a 96-well culture plate (NUNC, Roskilde, Denmark) for 24 h in a humidified atmosphere of 5% CO_2 and air at 37°C until subconfluence. After washing with Hanks' balanced salt solution (HBSS) three times, and preincubation with EBM-2 (BioWhittaker) for 12 h, the cells were stimulated with various doses of recombinant human (rh)-TNF-α, rh-IL-1β (both from ENDOGEN, Woburn, MA), lipopolysaccharide from *P. gingivalis* (kindly provided by H. Kumada and T. Umemoto, Department of Oral Microbiology, Kanagawa Dental University, Yokosuka, Japan), lipopolysaccharide from *E. coli* O111:B4 (List Biological Laboratories, Campbell, CA), *P. gingivalis* outer membrane protein (OMP), and *P. gingivalis* GroEL in EBM-2 supplemented with 10% fetal calf serum. The details of the preparation of *P. gingivalis* OMP (3) and GroEL (31) have been described previously.

Enzyme-linked immunosorbent assay (ELISA) for adhesion molecules

At the end of the culture period, the plates were washed with phosphate-buffered saline (PBS) three times and the cells were fixed with 4% phosphate-buffered paraformaldehyde for 15 min. After washing with PBS containing 0.05% Tween-20 (PBS-T; pH 7.4), nonspecific 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 biotinylated mouse anti-human CD54 (1 : 10000 dilution) and CD106 (1 : 1000 dilution; both from eBioscience, San Diego, CA) diluted with blocking reagent were added and incubated for 1 h at room temperature. After washing three times with PBS-T, horseradish peroxidase-conjugated avidin (eBioscience) diluted with blocking reagent was added and incubated for a further 45 min at room temperature. The o-phenylene diamine diluted to 1.3 mg/ml in 0.1 M citrate buffer containing 0.015% hydrogen peroxide was added. Color development was stopped by the addition of 1 M sulfuric acid and absorbance was read at a wavelength of 492 nm using an automated ELISA reader (Labsystems Oy, Helsinki, Finland) and analyzed using GENESIS LITE software (Labsystems).

Cytokine and chemokine assay

The levels of IL-6 and MCP-1 in the supernatants of HCAEC culture were determined by using commercially available ELISA kits (BioSource, Camarillo, CA) according to the manufacturer's instructions.

mRNA expression of TLR2 and TLR4 in HCAEC

Total RNA was isolated from unstimulated and stimulated HCAEC using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with RNase-free DNase I (Invitrogen). The RNA was then reverse-transcribed to cDNA using random primer (Takara Shuzo Co., Ltd., Shiga, Japan) and M-MLV reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) amplification of cDNA was performed using oligonucleotide primers specific for TLR2, TLR4 and GAPDH. The primer sequences are shown in Tabeta et al. (30). PCR amplification was performed with Taq DNA polymerase (Promega, Madison, WI) using a DNA thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, Foster, CA). The amplification cycle profile was as follows: for TLR2 and TLR4, denaturation at 94°C for 1 min; annealing at 58°C for 1 min; extension at 72°C for 1 min, for GAPDH, denaturation at 94°C for 1 min; annealing at 59°C for 1 min; extension at 72°C for 1 min. After amplification (30 cycles), each PCR product was electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. For real-time PCR, primers and probes were all purchased from Applied Biosystems and the sequences have been published previously (4, 21). Reactions were conducted in a 25 μl reaction mixture on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) following the predeveloped TaqMan assay reagents protocol (Applied Biosystems) and incubated for 10 min at 95°C, followed by 40 cycles

of a two-step amplification procedure composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. The ABI PRISM SDS 2.0 software (Applied Biosystems) was used to analyze the standards and quantifications.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data were analyzed by paired *t*-test. A probability value of < 0.05 was considered statistically significant.

Results

Induction of adhesion molecules on HCAECs by lipopolysaccharide

Lipopolysaccharide from *P. gingivalis* showed only a weak stimulatory effect on the expression of ICAM-1, and not on VCAM-1 on HCAEC. The effect was dose-dependent; however, statistically significant effects were observed at concentrations of 0.1 μg/ml and 1.0 μg/ml. The stimulatory effect of *E. coli* lipopolysaccharide was higher than that of *P. gingivalis* lipopolysaccharide. For VCAM-1 expression, no stimulatory effect of *P. gingivalis* lipopolysaccharide was observed. The effect of *E. coli* lipopolysaccharide on VCAM-1 expression was similar for ICAM-1 in which there was no stimulatory effect up to a concentration of 10 ng/ml but a significant increase in expression was shown at 0.1 μg/ml with a further increase at 1.0 μg/ml. The overall stimulatory effect of lipopolysaccharide was much lower than that of IL-1β and TNF-α (Fig. 1).

Induction of adhesion molecules on HCAECs by *P. gingivalis* outer membrane protein and GroEL

The stimulatory effect of *P. gingivalis* OMP and GroEL on the ICAM-1 and VCAM-1 was weak, and lower than that of *P. gingivalis* lipopolysaccharide. A statistically significant difference was observed only for *P. gingivalis* OMP at a concentration of 1 μg/ml. OMP and GroEL had little effect on VCAM-1 expression, however (Fig. 2).

Induction of adhesion molecules on HCAECs by TNF-α and IL-1β

In preliminary experiments, the maximum expression of adhesion molecules was observed after 24 h of stimulation; subsequent experiments were done at this incu-

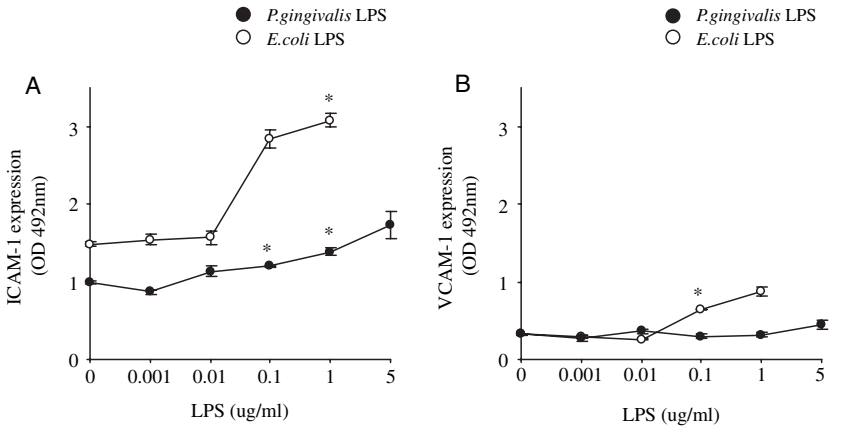

Fig. 1. P. gingivalis lipopolysaccharide- and *E. coli* lipopolysaccharide-induced expression of ICAM-1 (A) and VCAM-1 (B) in HCAECs. HCAECs were stimulated with various doses of the lipopolysaccharide for 24 h and the expression levels of adhesion molecules were determined by ELISA. Values represent the mean ± SEM of triplicate cultures. The experiments were repeated three times and representative data are shown. * $P < 0.05$ vs. control (paired *t*-test).

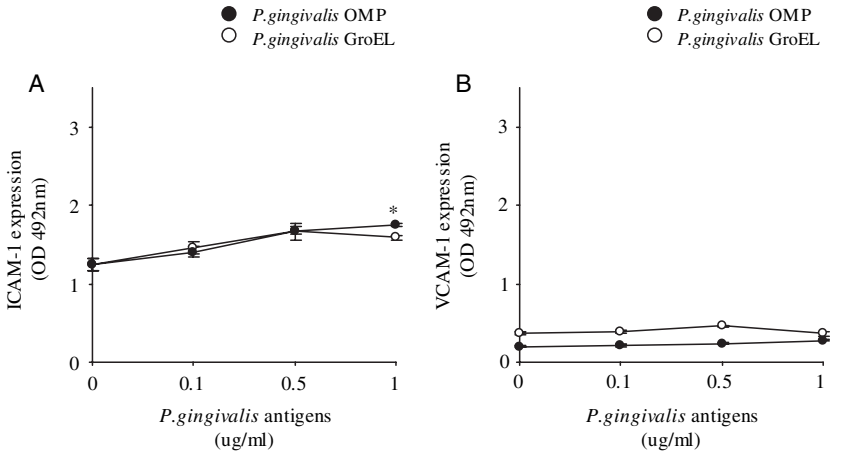

Fig. 2. P. gingivalis OMP- and *P. gingivalis* GroEL-induced expression of ICAM-1 (A) and VCAM-1 (B) in HCAECs. HCAECs were stimulated with various doses of the cytokines for 24 h and the expression levels of adhesion molecules were determined by ELISA. Values represent the mean ± SEM of triplicate cultures. The experiments were repeated at least three times and representative data are shown. * $P < 0.05$ vs. control (paired *t*-test).

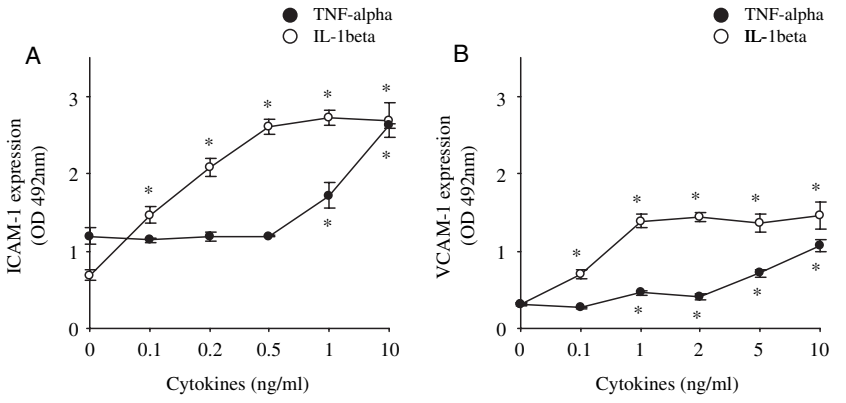

Fig. 3. TNF-α- and IL-1β-induced expression of ICAM-1 (A) and VCAM-1 (B) in HCAECs. HCAECs were stimulated with various doses of the cytokines for 24 h and expression levels of adhesion molecules were determined by ELISA. Values represent the mean ± SEM of triplicate cultures. The experiments were repeated three times and representative data are shown. * $P < 0.05$ vs. control (paired *t*-test).

bation time. As shown in Fig. 3, both TNF-α and IL-1β stimulated the expression of ICAM-1 on HCAEC in a dose-dependent manner, but the mode of action of the two cytokines was different. IL-1β showed a stimulatory effect at a concentration of 0.1 ng/ml and reached a maximum at 1.0 ng/ml. The apparent stimulatory effect for TNF-α, however, was observed at 1.0 ng/ml and rose further at 10.0 ng/ml. For VCAM-1 expression, again a stimulatory effect was observed at lower concentrations of IL-1β compared with TNF-α. Although the stimulatory effect of IL-1β was higher than that of TNF-α, VCAM-1 expression was much lower than ICAM-1 expression.

Inflammatory cytokines induce the production of IL-6 and MCP-1

HCAECs produce IL-6 and MCP-1 spontaneously. The basal levels of production were significantly increased by 24-h stimulation with TNF-α and IL-1β in a dose-dependent fashion. Although the effect of TNF-α and IL-1β on MCP-1 production was similar, the effect on IL-6 production was quite different inasmuch as the stimulatory effect of TNF-α was much lower than that of IL-1β (Fig. 4).

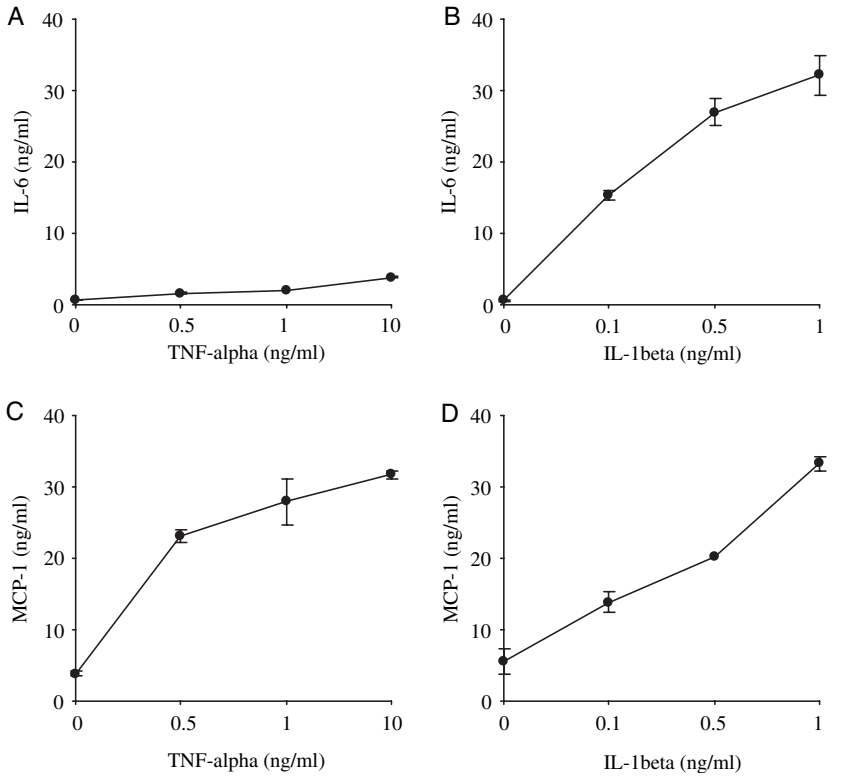

Fig. 4. TNF-α- and IL-1β-induced production of IL-6 (A and B) and MCP-1 (C and D) in HCAECs. HCAECs were stimulated with various doses of the cytokines for 24 h. The supernatants were collected and the levels of IL-6 and MCP-1 were determined by ELISA. Values represent the mean ± SEM of triplicate cultures.

E. coli lipopolysaccharide but not *P. gingivalis* antigens stimulate IL-6 and MCP-1 production

As shown in Fig. 5, lipopolysaccharide, OMP and GroEL derived from *P. gingivalis* did not enhance IL-6 and MCP-1 production by HCAECs. *E. coli* lipopolysaccharide, on the other hand, stimulated HCAECs to produce IL-6 and MCP-1 in a similar fashion to TNF-α.

Expression of TLR2 and TLR4 on HCAECs

Whereas HCAECs express TLR4 mRNA constitutively, mRNA for TLR2 could not be detected. Upon stimulation with TNF-α and IL-1β, the expression of mRNA for TLR2 was significantly up-regulated. The expression of mRNA for TLR4, however, appeared to decrease following IL-1β stimulation, as evidenced by image analysis. *E. coli* lipopolysaccharide, but not *P. gingivalis* lipopolysaccharide, up-regulated the mRNA for TLR2 slightly. The expression of mRNA for TLR4 was not affected either positively or negatively by lipopolysaccharide stimulation at the concentration tested (Fig. 6A). Real-time PCR confirmed the stimulatory effect of TNF-α, IL-1β and *E. coli* lipopolysaccharide on TLR2 expression by HCAEC (Fig. 6B). On the other hand, there was little effect of these stimulants on TLR4 expression (Fig. 6C).

Discussion

In contrast to the assumption that bacterial antigens in the bloodstream directly modulate endothelial function, resulting in aggravation of atherosclerosis, the present study showed little effect on the expression of adhesion molecules and the production of cytokines by HCAEC following stimulation with bacterial products. However, IL-1β and TNF-α, which are produced in a large amounts in periodontitis lesions, had significant effects on HCAECs.

Bacterial HSP60 not only induces pro-inflammatory cytokines and adhesion molecules in endothelial cells but also stimulates autoimmune responses against self-HSP60 as a result of molecular mimicry (25). *Chlamydia pneumoniae* has received much attention by cardiovascular researchers because of elevated levels of circulating antibodies to chlamydia HSP60 in CHD patients. These antibodies are thought to mediate endothelial cell cytotoxicity by reacting with autologous HSP60 on endothelial cells (20). In addition, Chlamydia HSP60 induces the production of proinflammatory cytokines such as TNF-α by endothelial cells, macrophages and smooth muscle cells (15). Although *P. gingivalis* HSP60 has also been demonstrated to stimulate adaptive immunity in which specific T-cells and antibody are elevated in periodontitis patients and patients with both periodontitis and atherosclerotic aneurysm, respectively (38, 39), this antigen failed to induce TNF-α production by monocyte-derived macrophages (32). However, little is known about the biological effects of HSP60 and other protein antigens derived from *P. gingivalis*, on endothelial cells. Although *P. gingivalis* HSP60 and OMP slightly up-regulated ICAM-1 expression, they had little effect on VCAM-1 expression and cytokine production. Therefore, the biological activity of *P. gingivalis* and *C. pneumoniae*, both of which are involved in common chronic inflammatory diseases, may be different.

In vitro studies clearly demonstrate that lipopolysaccharide, an active moiety of endotoxin, is a powerful inducer of adhe-

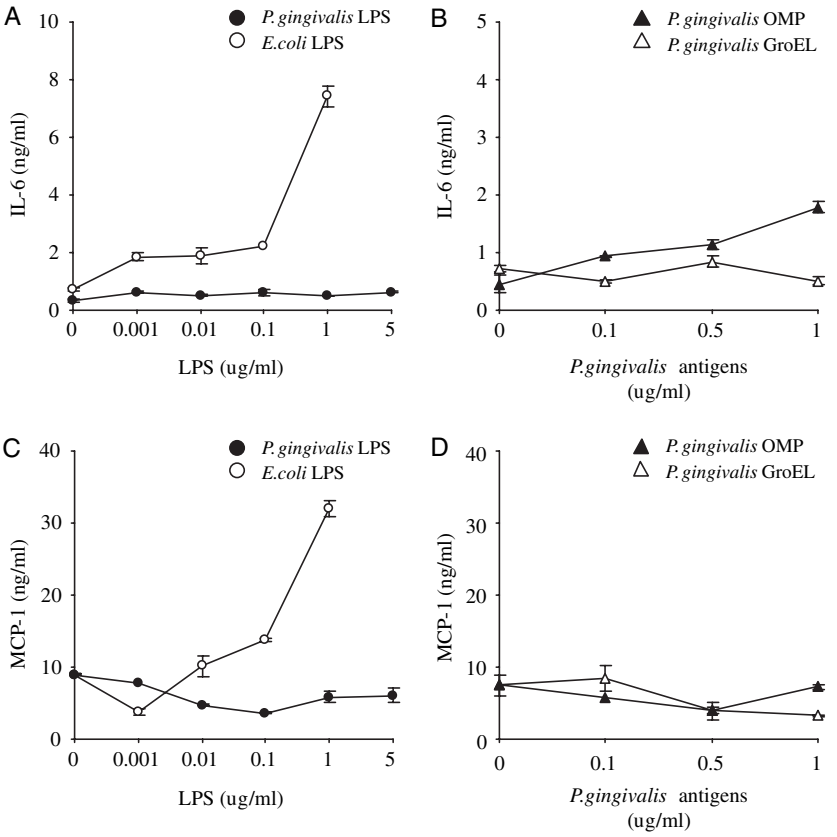

Fig. 5. Lipopolysaccharide- and *P. gingivalis* antigen-induced production of IL-6 (A and B) and MCP-1 (C and D) in HCAECs. HCAECs were stimulated with various doses of the antigens for 24 h. The supernatants were collected and the levels of IL-6 and MCP-1 were determined by ELISA. Values represent the mean ± SEM of triplicate cultures.

sion molecules on endothelial cells (13). In chronic periodontal infection, bacteria from the oral cavity can be released into the bloodstream in a variety of circumstances (11), for example during scaling (18), tooth extraction (22), and even toothbrushing (29). Once lipopolysaccharide and other bacterial components enter the bloodstream, cells expressing Toll-like receptors become activated. Ligation of these receptors initiates the activation of NF-κB, resulting in the expression of a wide array of inflammatory genes (19). In the present study, ICAM-1 expression was slightly up-regulated by stimulation with lipopolysaccharide from *P. gingivalis* and *E. coli*, but a statistically significant change was only observed at concentrations of 100 ng/ml or higher. The effect on VCAM-1 expression was similar, albeit to a much lesser extent. Concentrations of endotoxin >100 ng/ml can produce cellular activation independently of CD14 and TLR4, and thus may not accurately reflect how blood vessels might respond to clinically relevant levels of endotoxin (2).

As previously reported (7, 41), HCAECs constitutively express high levels of mRNA for TLR4, indicating that HCAECs are readily responsive to lipopolysaccharide. However, stimulation of HCAEC with TNF-α, IL-1β or lipopolysaccharide derived from *P. gingivalis* or *E. coli* did not change the expression of TLR4. In contrast, a previous study has shown that lipopolysaccharide stimulation up-regulated the mRNA expression of TLR4 in human microvascular endothelial cells (8), suggesting differential functions between these endothelial cells. On the other hand, in the present study, virtually no mRNA expression of TLR2 was observed in unstimulated HCAEC. However, upon stimulation with TNF-α and IL-1β at a concentration of 1.0 ng/ml, the mRNA expression of TLR2 was significantly up-regulated with a higher activity being seen with IL-1β. The effect of IL-1β was evident at a concentration as low as 1.0 pg/ml, which is comparable to the concentration seen in sera of periodontitis patients (data not shown). While as little as 10 ng/ml of *E. coli* lipopolysaccharide had an apparent stimulatory effect, 100 ng/ml lipopolysaccharide of *P. gingivalis* resulted in only a marginal elevation of TLR2 mRNA. This could also be attributable to the unique characteristics of *P. gingivalis* lipopolysaccharide.

In contrast to the bacterial antigens, relatively low levels of TNF-α and IL-1β can induce a substantial increase in ICAM-1 and VCAM-1 expression. Previous studies have already demonstrated these findings (1, 26); however, the concentration of cytokines used in this study to stimulate the cells was 10 ng/ml, which is much higher than 'physiological' levels seen in common chronic inflammatory diseases such as periodontitis. Ide et al. reported that the mean levels of IL-1β and TNF-α in the serum of chronic periodontitis patients before treatment were 0.95 pg/ml and 2.21 pg/ml, respectively (12). We also reported that the mean level of TNF-α in the serum of periodontitis patients was 1.90 pg/ml (37). IL-1β showed an apparent stimulatory effect on adhesion molecule expression at a concentration as low as 0.1 ng/ml. This concentration is still higher than that seen in the sera of periodontitis patients, but considering the dose-dependent response, the concentration of TNF-α of periodontitis patients is thought to have some effect on endothelial cell expression of adhesion molecules. In fact, TNF-α is reported to up-regulate the adherence of lymphocytes to endothelial cells at a concentration of 10 pg/ml (34).

Although some effects of *P. gingivalis* antigens and inflammatory cytokines on HCAEC functions were demonstrated, this does not indicate a causal relationship between periodontal infection and atherosclerosis. Most importantly, we utilized antigens from *P. gingivalis* but not other periodontopathic bacteria. Accumulating evidence clearly indicates that the total number of pathogens (pathogen burden) is a stronger and independent predictor of the presence and extent of coronary atherosclerosis (33). The concentrations of cytokines that induced a positive response were still higher than those in the sera of periodontitis patients, which in turn may reflect a systemic inflammatory response to the total periodontal infection. Whereas we can not comment on the relative importance of infection and inflammation, it should be noted that data from the present study do not preclude a role for periodontal infection in atherogenesis. The extent to which periodontal disease contributes to both the total burden of infection

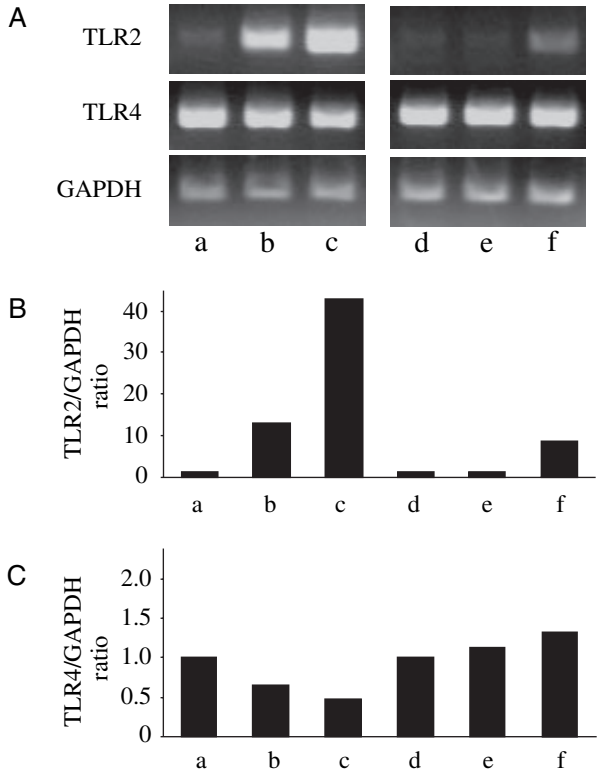

Fig. 6. Effects of lipopolysaccharide from *P. gingivalis* and *E. coli*, TNF-α and IL-1β on the expression of mRNA for TLR2 and TLR4. Expressions were analyzed by reverse-transcription-PCR (A). Quantitative analysis of the expression of TLR2 and TLR4 was conducted by real-time PCR (B and C). a, d): Control. b) TNF-α (1 ng/ml). c) IL-1β (1 ng/ml). e) *P. gingivalis* lipopolysaccharide (100 ng/ml). f) *E. coli* lipopolysaccharide (10 ng/ml).

and the total burden of inflammation needs to be further investigated.

Acknowledgments

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References

1. Amberger A, Maczek C, Jurgens G, Michaelis D, Schett G, Trieb K, et al. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. Cell Stress Chaperones 1997: **2**: 94–103.
2. Antal-Szalmas P. Evaluation of CD14 in host defence. Eur J Clin Invest 2000: **30**: 167–179.
3. Aoyagi T, Yamazaki K, Kabasawa-Katoh Y, Nakajima T, Yamashita N, Yoshie H, et al. Elevated CTLA-4 expression on CD4 T cells from periodontitis patients stimulated with *Porphyromonas gingivalis* outer membrane antigen. Clin Exp Immunol 2000: **119**: 280–286.
4. Claeys S, de Belder T, Holtappels G, Gevaert P, Verhasselt B, van Cauwenberge P, et al. Human β-defencins and toll-like receptors in the upper airway. Allergy 2003: **58**: 748–753.
5. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. Infect Immun 2004: **72**: 5041–5051.
6. DeStefano F, Anda RF, Kahn HS, Williamson DF, Russell CM. Dental disease and risk of coronary heart disease and mortality. BMJ 1993: **306**: 688–691.
7. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002: **105**: 1158–1161.
8. Faure E, Thomas L, Xu H, Medvedev AE, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-γ induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-κB activation. J Immunol 2001: **166**: 2018–2024.
9. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998: **2**: 275–281.
10. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 2001: **21**: 1876–1890.
11. Heimdahl A, Hall G, Hedberg M, Sandberg H, Soder PO, Tuner K, et al. Detection and quantitation by lysis-filtration of bacteremia after different oral surgical procedures. J Clin Microbiol 1990: **28**: 2205–2209.
12. Ide M, McPartlin D, Coward PY, Crook M, Lumb P, Wilson RF. Effect of treatment of chronic periodontitis on levels of serum markers of acute-phase inflammatory and vascular responses. J Clin Periodontol 2003: **30**: 334–340.
13. Jersmann HPA, Hii CST, Ferrante JV, Ferrante A. Bacterial lipopolysaccharide and tumor necrosis factor α synergistically increase expression of human endothelial adhesion molecules through activation of NF-κB and p38 mitogen-activated protein kinase signaling pathways. Infect Immun 2001: **69**: 1273–1279.
14. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med 2002: **347**: 185–192.
15. 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.
16. Lalla E, Lamster IB, Hofmann MA, Bucciarelli L, Jerud AP, Tucker S, et al. Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice. Arterioscler Thromb Vasc Biol 2003: **23**: 1405–1411.
17. Li L, Messas E, Batista EL Jr, Levine RA, Amar S. *Porphyromonas gingivalis* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation 2002: **105**: 861–867.
18. Lucartorto FM, Franker CK, Maza J. Post-scaling bacteremia in HIV-associated gingivitis and periodontitis. Oral Surg Oral Med Oral Pathol 1992: **73**: 550–554.
19. May MJ, Ghosh S. Signal transduction through NF-κB. Immunol Today 1998: **19**: 80–88.
20. Mayr M, Metzler B, Kiechl S, Willeit J, Schett G, 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.
21. Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, et al. Human intestinal epithelial cells are broadly unre-

sponsive to toll-like receptor 2-dependent bacterial ligands: Implications for host–microbial interactions in the gut. J Immunol 2003: **170**: 1406–1415.
22. Okabe K, Nakagawa K, Yamamoto E. Factors affecting the occurrence of bacteremia associated with tooth extraction. Int J Oral Maxillofac Surg 1995: **24**: 239–242.
23. Page RC. The pathobiology of periodontal diseases may affect systemic diseases: inversion of a paradigm. Ann Periodontol 1998: **3**: 108–120.
24. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO III, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: a statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003: **107**: 499–511.
25. Pockley AG. Heat shock proteins, inflammation, and cardiovascular disease. Circulation 2002: **105**: 1012–1017.
26. Raab M, Daxecker H, Markovic S, Karimi A, Griesmacher A, Mueller MM. Variation of adhesion molecule expression on human umbilical vein endothelial cells upon multiple cytokine application. Clin Chim Acta 2002: **321**: 11–16.
27. Rice JB, Stoll LL, Li WG, Denning GM, Weydert J, Charipar E, et al. Low-level endotoxin induces potent inflammatory activation of human blood vessels: inhibition by statins. Arterioscler Thromb Vasc Biol 2003: **23**: 1576–1582.
28. Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med 1999: **340**: 115–126.
29. Silver JG, Martin AW, McBride BC. Experimental transient bacteraemias in human subjects with varying degrees of plaque accumulation and gingival inflammation. J Clin Periodontol 1977: **4**: 92–99.
30. Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, et al. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. Infect Immun 2000: **68**: 3731–3735.
31. 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.
32. Ueki K, Tabeta K, Yoshie H, Yamazaki K. Self-heat shock protein 60 induces tumour necrosis factor-α in monocyte-derived macrophage: possible role in chronic inflammatory periodontal disease. Clin Exp Immunol 2002: **127**: 72–77.
33. Vita JA, Loscalzo J. Shouldering the risk factor burden: infection, atherosclerosis, and the vascular endothelium. Circulation 2002: **106**: 164–166.
34. Watson C, Whittaker S, Smith N, Vora AJ, Dumonde DC, Brown KA. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. Clin Exp Immunol 1996: **105**: 112–119.
35. Wenzel RP, Pinsky MR, Ulevitch RJ, Young L. Current understanding of sepsis. Clin Infect Dis 1996: **22**: 407–412.
36. Wood D. Established and emerging cardiovascular risk factors. Am Heart J 2001: **141**: S49–S57.
37. Yamazaki K, Honda T, Oda T, Ueki-Maruyama K, Nakajima T, Yoshie H, et al. Effect of periodontal treatment on the C-reactive protein and proinflammatory cytokine levels in Japanese periodontitis patients. J Periodontal Res 2005: **40**: 53–58.
38. Yamazaki K, Ohsawa Y, Itoh H, Ueki K, Tabeta K, Oda T, et al. T-cell clonality to *Porphyromonas gingivalis* and human heat shock protein 60s in patients with atherosclerosis and periodontitis. Oral Microbiol Immunol 2004: **19**: 160–167.
39. Yamazaki K, Ohsawa Y, Tabeta K, Itoh H, Ueki K, Oda T, 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.
40. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 2000: **148**: 209–214.
41. Zeuke S, Ulmer AJ, Kusumoto S, Katus HA, Heine H. TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. Cardiovasc Res 2002: **56**: 126–134.