Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 402–408

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01441.x

Short communication

Oral infection with *Porphyromonas gingivalis* and systemic cytokine profile in C57BL/6.KOR-*ApoE*^{shl} mice

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Miyauchi S, Maekawa T, Aoki Y, Miyazawa H, Tabeta K, Nakajima T, Yamazaki K. Oral infection with Porphyromonas gingivalis *and systemic cytokine profile in C57BL/6.KOR-*ApoE^{shl} *mice. J Periodont Res 2012; 47: 402–408. © 2011 John Wiley & Sons A/S*

Background and Objective: Periodontal infection affects atherosclerotic diseases, such as coronary heart diseases. Mouse models have revealed that oral infection with *Porphyromonas gingivalis* induces changes in inflammatory- and lipid metabolism-related gene expression, regardless of the development of atherosclerotic lesions. However, the serum protein expression profile in the oral infection model has not been investigated. The present study aimed to analyse the effect of oral infection with *P. gingivalis* on the expression levels of multiple cytokines in the serum in apolipoprotein E-deficient mice by using a cytokine antibody array.

Material and Methods: C57BL/6.KOR-*Apoe*^{shl} mice were orally infected with *P. gingivalis* five times at 3 day intervals and were then killed. Splenocytes were isolated and analysed for proliferative activity and immunoglobulin G (IgG) production in response to *in vitro* restimulation with *P. gingivalis*. The expression levels of various cytokines in the sera were analysed using a mouse antibody array glass chip.

Results: Splenocytes from *P. gingivalis*-infected mice demonstrated significantly greater proliferation and IgG production in response to *P. gingivalis* compared with those from sham-infected mice. Antibody array analysis revealed the selective upregulation of matrix metalloproteinase 3, intercellular adhesion molecule 1, insulin-like growth factor binding protein 2 and chemokine (C-X-C motif) ligand 7 and the downregulation of interleukin-17, tumor necrosis factor-α and L-selectin.

Conclusion: These data demonstrate that oral infection with *P. gingivalis* induces alterations in systemic cytokine production. These cytokines could play roles in the development not only of periodontitis but also of atherosclerosis.

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Key words: animal model; cytokine; periodontal medicine; *Porphyromonas gingivalis*

Accepted for publication October 16, 2011

Recent evidence suggests that periodontal infection not only induces periodontal tissue destruction but also adversely affects systemic health (1). The conditions affected by periodontal disease include atherothrombotic diseases, such as coronary heart disease, and cerebrovascular disease, diabetes and preterm birth. Recent studies suggest that certain chronic infections may be considered novel and potentially modifiable risk factors (2). A number

of molecules have been identified as risk factors and risk markers for atherosclerosis. In particular, acute-phase proteins, such as C-reactive protein and serum amyloid A (SAA), have received considerable attention because elevated levels of these molecules not only were observed in coronary heart disease patients but also predicted future development of the disease. Furthermore, in addition to the induction of acute-phase proteins, chronic infection status conferred a markedly increased risk of atherosclerosis development, even in the absence of other vascular risk factors (3).

Our previous study demonstrated that oral infection with *Porphyromonas gingivalis* in ApoE-deficient C57BL/6.KOR-*Apoe^{shl}* (B6.Apoeshl) mice clearly induced the elevation of SAA (the mouse equivalent of human C-reactive protein), with a concomitant increase in the number of atherosclerotic lesions (4). Gene expression analysis clarified that the expression of the molecules related to inflammation was elevated in the aorta, and the expression of lipid metabolism-related genes, particularly those involved in cholesterol efflux, was impaired in the liver. As direct invasion of bacteria into the systemic circulation was not observed in this model, changes in gene expression could be attributable to immune and/or inflammatory responses to the organism.

In this regard, the B6.Apoeshl mouse is a useful model for investigating the effect of the periodontitis-induced systemic inflammatory response on atherogenesis, because the atherosclerotic lesion that is accelerated by oral infection with *P. gingivalis* develops in B6.Apoeshl mice but not in wild-type mice (4).

Animal studies that have attempted to elucidate the effect of periodontal infection on a systemic condition have mainly examined the pathological changes, the inflammatory markers (5–7) and gene expression (4,8) in the aorta and liver. To the best of our knowledge, there are no data on the protein profile in the oral infection model. Almost all cell functions are executed by proteins, which cannot be studied by examining DNA and RNA alone. Experimental analysis clearly shows a disparity between the relative expression levels of mRNAs and their corresponding proteins (9); therefore, it is critical to analyse the protein profile.

To gain further insight into the mechanisms by which periodontal infection affects systemic conditions, we analysed the effect of *P. gingivalis* oral infection on the expression levels of multiple cytokines in the serum of B6.Apoeshl mice by using a cytokine antibody array.

Material and methods

Mice

All animal studies were performed in accordance with the policies of the Institutional Animal Care and Use Committee at Niigata University. Six-week-old male C57BL/6 (wild-type) mice and spontaneously hyperlipidemic male B6.Apoeshl mice (10,11) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were maintained in specific pathogen-free conditions and fed regular chow and sterile water until the commencement of infection at 8 wk of age.

Bacterial culture

P. gingivalis strain W83 was cultured in modified Gifu Anaerobic Medium (GAM) broth (Nissui, Tokyo, Japan) in an anaerobic jar (Becton Dickinson Microbiology System, Cockeysville, MD, USA) in the presence of an AnaeroPack^{TM} (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) for 48 h at 37°C. Bacterial suspensions were prepared in phosphate-buffered saline without Mg^{2+}/Ca^{2+} using established growth curves and spectrophotometric analysis. The number of colony forming units was standardized using optical density (600 nm).

Oral infection

The murine experimental periodontitis model was developed according to Baker *et al.* (12), with slight modifications. The animals received the antibiotics sulfamethoxazole and trimethoprim at final concentrations of 700 and 400 μg/mL, respectively, in water bottles *ad libitum* for 10 d, and this treatment was followed by 3 d without antibiotics. The experimental group was then infected. A total of 10^9 colony forming units of live *P. gingivalis* suspended in 100 μL of phosphate-buffered saline with 2% carboxymethyl cellulose (Sigma-Aldrich, St Louis, MO, USA) was given to each mouse via a feeding needle. This suspension was given five times at 3 d intervals. The control group received the same pretreatment and was sham infected without the *P. gingivalis*. Two days after the final treatment, the mice were killed by CO_2 inhalation, and their tissues were removed.

Serum SAA levels

Blood samples were collected by cardiac puncture before killing, and 200 μL of serum were separated after centrifugation, and the SAA was measured using a commercial ELISA kit (Invitrogen, Carlsbad, CA, USA).

Proliferation of spleen cells and production of immunoglobulin G and interleukin-6

The proliferation and immunoglobulin G (IgG) production of splenocytes isolated from *P. gingivalis*-infected and sham-infected mice were assayed by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and ELISA (Pierce Biotechnology, Rockford, IL, USA), respectively, according to the manufacturers' instructions. Briefly, cells (5×10^5 per well) were stimulated with the soluble antigen of *P. gingivalis* W83 (Institute of Immunology Co., Ltd, Tokyo, Japan) at concentrations of 100 and 1000 ng/mL for 2, 4 and 6 d. At each time point, aliquots (70 μL) were removed for cytokine ELISAs, and the residual cultures were used for cell counting. Phytohemagglutinin (PHA)-P (Sigma-Aldrich, St Louis, MO, USA) was used at a concentration of 10 μg/mL and served as a positive control.

Levels of IgG specific for *P. gingivalis* W83 in the culture supernatant were determined by ELISA, as described previously (4), and interleukin (IL)-6 levels were determined by an

ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA).

Antibody array

The expression levels of various cytokines in the sera from infected and sham-infected mice were analysed using a mouse antibody array glass chip (RayBio Mouse Cytokine Antibody Array G series 1000; RayBiotech Inc., Norcross, GA, USA). Incubation and washes were performed according to the manufacturer's instructions. Briefly, chip arrays were blocked at room temperature for 30 min before being incubated with 150 μL of each sample at room temperature for 16 h. Glass chips were then washed and incubated with biotin-conjugated primary antibody and fluorescent dye-conjugated streptavidin according to the manufacturer's instructions. Fluorescence detection and analysis were performed using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA, USA). A list of the cytokines examined is shown in Table 1.

Statistical analysis

GRAPHPAD Prism (GraphPad Software, Inc., La Jolla, CA, USA) was used for the analysis. Parametric data were analysed using Student's paired or unpaired *t*-test. Nonparametric data were analysed by Mann–Whitney *U*-test. A probability value of < 0.05 was considered statistically significant.

Results and Discussion

Oral infection with *P. gingivalis* induced a significant elevation in the serum SAA level compared with sham-infected mice ($p < 0.01$; Fig. 1). Furthermore, splenocytes from *P. gingivalis*-infected mice demonstrated significantly greater proliferation (Fig. 2A), as well as IgG (Fig. 2B) and IL-6 production (Fig. 2C) in response to *in vitro* restimulation with *P. gingivalis* antigens compared with those from sham-infected mice.

These data suggest that oral inoculation with *P. gingivalis* induces systemic inflammation and modulation of the immune response without apparent bacteremia (data not shown). This conclusion is consistent with additional data showing that the selective upregulation or downregulation of several molecules in the sera of infected mice was detectable on the microarray membranes. Among the examined molecules listed in Table 1, matrix metalloproteinase 3 (MMP-3), intercellular adhesion molecule 1 (ICAM-1),

Table 1. List of 96 examined cytokines

Axl
B lymphocyte chemoattractant (BLC) (CXCL13)
Basic fibroblast growth factor (bFGF)
CD30
CD30 ligand
CD40
Cytokine-responsive gene (CRG) -2 (CXCL10)
Cutaneous T cell attracting chemokine (CTACK) (CCL27)
CXCL16
Dipeptidyl peptidase (DPP) IV/CD26
Dtk
E-Selectin
Eotaxin (CCL11)
Eotaxin-2 (CCL24)
Fas ligand
Fcγ receptor IIB
Fms-related tyrosine kinase 3 (Flt-3) ligand
Fractalkine (CX3CL1)
Glucocorticoid-induced TNF-receptor (GITR) (TNFRSF18)
Granulocyte colony stimulating factor (GCSF)
Granulocyte-macrophage colony-stimulating-factor (GM-CSF)
Hepatocyte growth factor (HGF) receptor
Intercellular adhesion molecule (ICAM) -1
Interferon γ
Insulin-like growth factor binding protein (IGFBP) -2
IGFBP-3
IGFBP-5
IGFBP-6
Insulin-like growth factor (IGF) -I
IGF-II
Interleukin (IL) -1α
IL-1β
IL-2
IL-3
IL-3 receptor β
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-12 p40/p70
IL-12 p70
IL-13
IL-15
IL-17
IL-17B receptor
Interferon-inducible T-cell alpha chemoattractant (I-TAC) (CXCL11)
Keratinocyte-derived chemokine (KC) (CXCL1)
Leptin
Leptin receptor
LPS-inducible CXC chemokine (LIX) (CXCL5)
Lungkine (CXCL15)
L-Selectin
Lymphotactin (XCL-1)
Monocyte chemoattractant protein (MCP) -1 (CCL2)
MCP-5 (CCL12)
Macrophage colony-stimulating factor 1 (M-CSF)
Macrophage-derived chemokine (MDC) (CCL22)
Monokine induced by gamma interferon (MIG) (CXCL9)
Macrophage inflammatory protein (MIP) -1α (CCL3)
MIP-1γ (CCL9)
MIP-2 (CXCL2)
MIP-3α (CCL20)
MIP-3β (CCL19)
Matrix metalloproteinase (MMP) -2
MMP-3
Osteopontin
Osteoprotegerin
Platelet factor (PF) -4 (CXCL4)
P-Selectin
Pro-MMP-9
Regulated upon activation normal T-cell expressed and secreted (RANTES) (CCL5)
Resistin
Stem cell factor (SCF)
Stromal cell-derived factor (SDF) -1α (CXCL12)
Sonic hedgehog (Shh) -N
Thymus and activation-regulated chemokine (TARC) (CCL17)
T-cell activation protein (TCA) -3 (CCL1)
Thymus expressed chemokine (TECK) (CCL25)
Thymus CK-1 (CXCL7)
Tissue inhibitor of metalloproteinase (TIMP) -1
TIMP-2
Tumor necrosis factor (TNF) -α
Soluble TNF (sTNF) receptor I
sTNF receptor II
Thyroid peroxidase (TPO)
TNF-related activation-induced cytokine (TRANCE) (TNFSF11)
TROY (TNFRSF19)
Thymic stromal lymphopoietin (TSLP)
Vascular cell adhesion molecule (VCAM) -1
Vascular endothelial growth factor (VEGF)
VEGF receptor 1
VEGF receptor 2
VEGF receptor 3
VEGF-D

Fig. 1. Effects of oral infection with *P. gingivalis* on serum levels of serum amyloid A (SAA). All experiments were performed in triplicate wells. Significant differences were observed between the *P. gingivalis*-infected group and the sham-infected group ($n = 5$; $^*p < 0.01$, Mann–Whitney *U*-test).

insulin-like growth factor binding protein 2 (IGFBP-2) and thymus CK-1 (hereafter designated as CXCL7) were the molecules that were significantly upregulated, whereas IL-17, tumor necrosis factor α (TNF-α) and L-selectin were significantly downregulated in the infected mice (Table 2).

The levels of soluble ICAM-1 (sICAM-1) were elevated in the sera of *P. gingivalis*-infected B6.Apoeshl mice. This finding is consistent with the findings in patients with coronary artery disease (13) and implies endothelial cell, smooth-muscle cell and monocyte activation. Elevated levels of sICAM-1 may be involved in the acceleration of atherosclerotic lesion development.

CXCL7, the most abundant platelet chemokine (14), was also elevated in the infected mice. A proteolytic product of CXCL7 called neutrophil activating peptide 2 has been demonstrated to bind to chemokine (C-X-C motif) receptor (CXCR) 1 and CXCR2 (15) and to induce neutrophil adhesion to human umbilical vein endothelial cells in a dose-dependent manner *in vitro* (16). Although there is little direct evidence of a proatherogenic role for CXCL7, it is conceivable that periodontal infection is important for creating an inflammatory environment by inducing CXCL7 expression.

IGFBP-2 has been shown to play a protective role against the development of insulin resistance and obesity (17). As inflammation can promote insulin resistance and dysregulate glycemia, periodontal disease is supposed to induce insulin resistance. Although the adverse effects of periodontitis on glycemic control have not been proved, our previous study demonstrated that adiponectin receptor 2 (AdipoR2), a specific receptor for adiponectin, was downregulated in the livers of infected mice (4). This finding is of particular interest because the targeted disruption of AdipoR2 resulted in decreased activity of the peroxisome proliferator-activated receptor γ signaling pathway and in insulin resistance (18). Therefore, it is likely that IGFBP-2 is elevated to promote insulin resistance.

MMP-3 plays a pivotal role in extracellular matrix degradation in periodontal tissue during periodontopathic bacteria-induced pathogenesis. In addition, it is reported that plasma MMP-3 levels are an independent prognostic factor in coronary artery disease (19).

In contrast, plasma concentrations of TNF-α have been positively correlated with elevated plasma triglycerides and heart failure (20). In addition, TNF-α is indirectly involved in coronary heart disease risk through an increase in insulin resistance. In this respect, the downregulation of TNF-α in the infected mice compared with sham-infected mice seems to contradict the finding described above. While plasma TNF-α levels are increased in periodontitis patients compared with periodontally healthy subjects (21), these levels are increased after periodontal therapy (22). In contrast, we demonstrated that serum TNF-α levels of periodontitis patients were lower than those of control subjects. The levels were increased after successful periodontal treatment (23,24). Therefore, the downregulation of TNF-α in infected mice is consistent with the findings seen in human periodontitis patients.

Interleukin-17 plays an important pathological role in periodontal disease. Increased levels of IL-17 mRNA and protein are reported in the gingival tissue and gingival crevicular fluid of periodontitis patients (25–31). Levels of IL-17 are increased in the sera of aggressive periodontitis (32) but not of chronic periodontitis patients (33). However, the role of IL-17 in atherosclerosis remains controversial, with different studies suggesting either a proatherogenic or an atheroprotective role (34–37). Unlike human periodontitis patients, in the B6.Apoeshl mice oral infection with *P. gingivalis* downregulated the serum IL-17 levels. We are the first to demonstrate infection-induced systemic levels of IL-17 in the context of periodontal disease in mice. Although the underlying mechanisms for this downregulation are not known, given that IL-17 is protective against *P. gingivalis*-induced bone loss in a murine model (38), *P. gingivalis* oral infection may impair defense systems in hyperlipidemic conditions.

L-Selectin is a leukocyte antigen that appears to be responsible for the initial attachment of leukocytes to the endothelium and that is rapidly shed from neutrophils after chemotactic stimulation. Unlike acute inflammatory illness, patients with chronic inflammatory diseases have been shown to have lower than normal levels (39). Consistent with this finding, it is reported that patients with ischemic heart disease have lower serum soluble L-selectin (sL-selectin) levels than healthy subjects (13). Furthermore, low levels of sL-selectin have been observed in plasma from periodontitis patients (40). Decreased sL-selectin levels may reflect the sequestration of sL-selectin by widespread binding to activated endothelium.

Collectively, the present study clearly demonstrated that oral infection with *P. gingivalis* induces alteration of systemic cytokine levels, which plays a role not only in periodontal tissue destruction but also in atherogenesis in B6.Apoeshl mice. These data further support the evidence that periodontitis is associated with systemic diseases.

Acknowledgements

The authors thank Dr Yoshibumi Matsushima for kind instruction on the use of C57BL/6.KOR -*Apoe*^{shl} mice. This study was supported by the Japan Society for the Promotion of Science (23390476 and 23659974 for K.Y., 20295426 for K.T. and 21390555 for T.N., and the Young Researcher

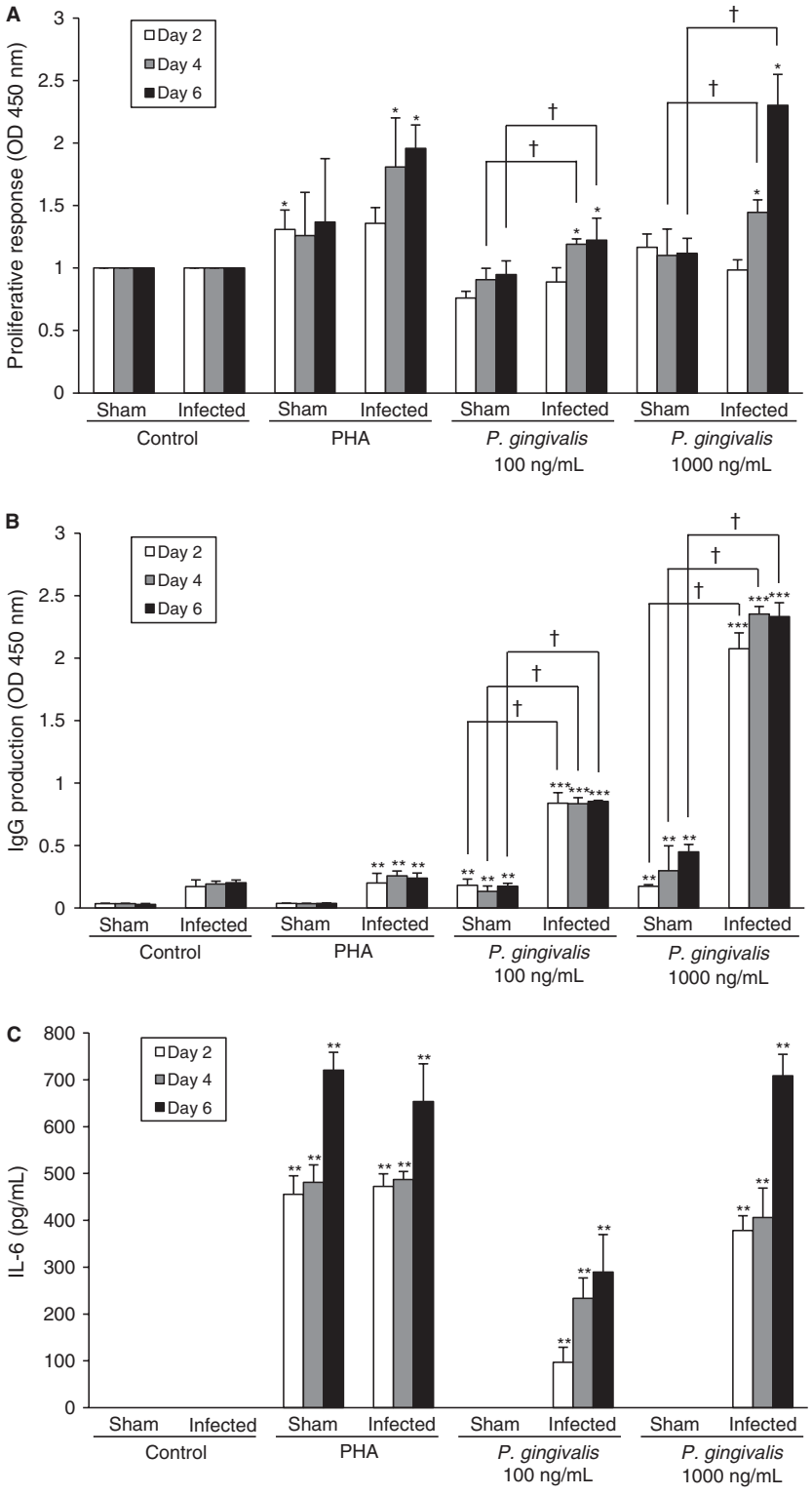

Fig. 2. Spleen cell response to *P. gingivalis* antigen. Spleen cells were stimulated with *P. gingivalis* antigen. The proliferative response (A), production of immunoglobulin G (IgG; B) and production of IL-6 (C) were compared between the sham-infected group and the *P. gingivalis*-infected group. The results are expressed as the means + SD of three mice per group. Differences in the effects of the control, phytohemagglutinin (PHA) and *P. gingivalis* antigen were analysed by Student's paired *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Differences between the sham-infected and *P. gingivalis*-infected animals were analysed by Student's unpaired *t*-test (†$p < 0.05$).

Table 2. Relative cytokine levels in the sera of infected mice

Cytokine	Average fluorescence intensity		Ratio (b/a)	*p*-Value
	Sham-infected[(a)]	Infected[(b)]		
IL-17	7802.53	6094.29	0.78	0.012
L-Selectin	31498.42	23658.68	0.75	0.041
TNF-α	3265.67	2595.45	0.79	0.029
ICAM-1	977.17	1537.91	1.57	0.048
IGFBP-2	1739.88	2271.92	1.31	0.032
MMP-3	10425.64	17316.68	1.66	0.019
Thymus CK-1 (CXCL7)	24463.87	29526.76	1.21	0.009

Overseas Visits Program for Vitalizing Brain Circulation, S2203) and the Promotion of Niigata University Research Project.

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