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Periodontitis May Increase the Risk of Peripheral Arterial Disease

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Objectives. *The aim of this case control study was to evaluate whether periodontitis was associated with peripheral arterial disease (PAD).*
Subjects and Methods. *Twenty-five patients diagnosed with aorto-iliac and/or femoro-popliteal occlusive disease and thirty-two generally healthy control subjects were enrolled in this study. Polymerase chain reaction (PCR) was used to identify* Porphyromonas gingivalis, Treponema denticola, Actinobacillus actinomycetemcomitans, Prevotella intermedia, *Cytomegalovirus (CMV),* Chlamydia pneumoniae, *and* Helicobacter pylori *in tissue specimens taken from the anastomotic site of distal bypasses. Periodontal status was evaluated; serum IgG titres against the four listed bacteria were measured.*
Results. *Periodontopathic bacteria were detected in 13/25 (52%) atherosclerotic specimens. CMV or* C. pneumoniae *was detected in 1/25 (4%) specimens;* H. pylori *was not detected from any of these specimens. Fontaine grade III or IV patients showed higher detection frequency of* P. gingivalis *than Fontaine grade II patients (57.1% vs 22.2%,* $P = 0.09$*). After adjusting for age, gender, diabetes and smoking, periodontitis increased 5-fold the risk of having PAD (OR 5.45). There were preliminary indications that periodontitis was associated with increased serum IL-6 and TNF-α concentrations.*
Conclusions. *This study suggests that periodontitis may be associated with an increased risk of PAD. This association could result from the increased concentration of serum inflammatory cytokines in those with periodontitis.*

Keywords: PAD; Atherosclerosis; Periodontitis; Inflammatory cytokines; P. gingivalis; T. denticola.

Introduction

Peripheral arterial disease (PAD), mostly associated with atherosclerosis, results in obstruction to blood flow and lower extremity ischemic ulceration or gangrene, with amputation eventually being required in up to 20 to 25% of these patients.[1] PAD shares the common underlying pathology of atherosclerosis with other cardiovascular diseases including stroke.[2] It has been reported PAD patients have higher circulating levels of IL-6 and TNF-α.[3] Elevated vascular inflammatory markers including IL-6 and TNF-α are suggested to be associated with the extent of atherosclerosis in PAD patients.[4]

Periodontitis is a chronic inflammatory disease of tooth-supporting tissues caused by periodontopathic bacteria.[5] Periodontitis patients are reported to have higher serum IL-6, CRP and neutrophils in several studies.[6–8] Serum IL-6 and CRP levels also show a positive relation to the extent of periodontitis.[7] Serum TNF-α and IL-1β have been reported to be significantly higher in periodontitis patients than healthy controls.[9] These results suggest that periodontitis, as a chronic inflammatory condition, may contribute to increased serum inflammatory cytokines.

The relationship between periodontitis and atherosclerosis has been assumed since the initial observations that periodontal pathogens were identified from atheromatous plaques.[10,11] Epidemiological

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studies during the past decade suggest that periodontitis increases the risk of stroke and coronary heart diseases.[12–16] There is far less information about any possible association between dental diseases and PAD. Mendes and colleagues first reported that subjects with clinical significant periodontal diseases at baseline had a 2.27 fold greater risk for developing PAD (OR = 2.27, 95% CI = 1.32-3.9).[17] Hung *et al*. conducted a prospective cohort study ($n = 342$) and found that periodontal diseases were associated with a relative risk of 1.41 (95% CI = 1.12-1.77) for developing PAD during a 12-year follow up.[18] These two epidemiological studies described the possible relation between periodontitis and PAD, but still little is known through which route periodontitis may have influence on PAD. In our previous studies, multiple periodontopathic bacteria frequently were detected from the diseased arteries of patients with abdominal aortic aneurysms or Buerger's disease.[19–21] Hence periodontitis may be associated with multiple vascular complications.

The aim of this case control study was to evaluate whether periodontitis is associated with PAD. In the present study, we investigated the localization of periodontopathic bacteria in atherosclerotic specimens. Periodontal status was evaluated; in addition, serum IgG titres against periodontopathic bacteria and serum inflammatory cytokines including IL-6, TNF-α and IL-1β were examined in PAD patients and a control group.

Subjects and Methods

Study population

Twenty-five PAD patients diagnosed with aorto-iliac and/or femoro-popliteal occlusive disease were recruited from the Clinic of Vascular and Applied Surgery in Tokyo Medical and Dental University. PAD was diagnosed based on clinical symptoms, ankle brachial pressure index (ABI), and angiographic findings.[1] The stage of disease was described as Fontaine grade based on clinical symptoms: 18 patients were Fontaine grade II (intermittent claudication), 5 patients were Fontaine grade III (rest pain), and 2 patients were Fontaine grade IV (ischemic ulceration and gangrene). Fontaine grade III or IV patients (seven patients) also were diagnosed as having critical limb ischemia. The ABI of PAD patients ranged from 0 to 0.7, mean 0.54. Fourteen patients had supra-inguinal occlusions, 6 patients had infra-inguinal occlusions and 5 patients had combined lesions. All patients had TASC type C or type D lesions and underwent bypass surgery (rather than endovascular treatment), and a tissue sample from the anastomotic site (anterior wall) was obtained for PCR analysis.

Thirty-two generally healthy control subjects, defined as those without atherosclerosis and normal ABI, were matched for age, gender, race and smoking status. Information on current health status, medical history, drug use and smoking behavior was obtained with the use of a questionnaire during an interview. Subjects were excluded if they had received antibiotics within the previous 3 months or treatment for periodontal disease within 6 months of the study. All subjects provided informed consent and the study was approved by the Ethical Committee of Tokyo Medical and Dental University.

Clinical periodontal examinations

Periodontal status was evaluated using various clinical parameters by a trained periodontist, who was blinded to clinical symptoms of PAD patients. Periodontal probing depth (PD) and clinical attachment level (CAL) were recorded at 6 points of each tooth. CAL was calculated from the sum of probing depth and gingival recession. The definition of periodontitis was modified from the Atherosclerosis Risk in Communities (ARIC) dental examinations described by Beck *et al*.[22] Participants who presented with at least one probing site with PD $\geq$ 4 mm or CAL $\geq$ 4 mm in each quadrant were defined as periodontitis patients. The number of residual teeth was also recorded. Periodontal examinations were performed before PAD patients received surgical operations.

Detection of periodontopathic bacteria, Cytomegalovirus (CMV), Chlamydia pneumoniae and Helicobacter pylori in arterial specimens

25 fresh and sterile atherosclerotic specimens approximately 1.5 cm long were obtained during surgery and stored immediately at -80°C until use. After specimens were homogenized, a High-Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) was used to extract DNA from the homogenized tissues. PCR (as described previously[23]) was used to detect four specific periodontopathic bacteria, namely *Porphyromonas gingivalis*, *Treponema denticola*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia*. Briefly, 5 μl of sample was added to 45 μl of reaction mixture containing 5 μl 10xPCR buffer (Promega, Madison, WI), 1.25 unit Taq DNA polymerase (Promega), and 0.2 mM of each of the deoxyribonucleotides (Pharmacia LKB, Piscataway, NJ). The optimal

$MgCl_2$ concentration in the mixture was 1.5 mM for *P. gingivalis* and *T. denticola,* and 1.0 mM for *A. actinomycetemcomitans* and *P. intermedia.* PCR amplification was performed in a DNA thermal cycler (PTC-100, MJ research, Boston, MA). The detection of CMV was performed by artus® CMV LC PCR Kit (Roche, Hamburg, Germany); the detection of *C. pneumoniae* was performed by AMS25 (CLONIT S.r.l. Milano, Italy); and the detection of *H. pylori* was performed by MPCR kit (Maxim Biotech, Inc. San Francisco, CA, USA) according to the manufacturer's instructions, respectively.

Determination of Serum IgG antibody titers

Whole blood samples were taken from all study subjects at the time of dental visit. Blood samples were centrifuged at 2500 rpm for 10 min at 4°C. The serum was filtered and immediately stored at −80°C until analysis. Serum IgG titers against *P. gingivalis*, *T. denticola, A. actinomycetemcomitans* and *P. intermedia* were measured using a previously described ELISA method.[24]

Measurement of serum inflammatory cytokines

Serum IL-6, TNF-α, and IL-1β were measured using high-sensitivity quantitative sandwich enzyme immunoassay technique (Quantikine® HS; R&D Systems International, Minneapolis, USA) according to the manufacturer's instructions. The minimum detectable dose was 0.016 pg/ml for IL-6, 0.06 pg/ml for TNF-α, and 0.1 pg/ml for IL-1β.

Statistical analysis

Descriptive statistics and statistical analyses were performed with a computerised statistical package (SPSS). The Kolmogorov-Smirnov normality test and the Levene variance homogeneity test were applied to examine the distribution normality of the data. Statistical differences by gender, smoking status and periodontitis prevalence between the two groups were tested by Fisher's exact test. The detection frequency between Fontaine grade II patients and Fontaine grade III or IV patients was also compared by Fisher's exact test. Mann-Whitney U test was applied to compare the differences in age, percentages of probing sites with PD and CAL, number of residual teeth, and serum IgG antibody titres. In addition, Mann-Whitney U test was used to compare the serum cytokine levels in PAD patients with periodontitis and PAD patients without periodontitis. The same comparison was performed in the control subjects with periodontitis and control subjects without periodontitis. Logistic regression was applied to test the association between periodontitis and PAD in all subjects adjusting for multiple confounding factors including smoking, age, gender and diabetes. Statistical significance was set at $P < 0.05$.

Results

Characteristics of participants

The demographics of the PAD patients and control subjects are shown in Table 1. There were no significant differences regarding age, gender and smoking status.

Periodontal examinations

The prevalence of periodontitis, and percentage of probing sites with $PD \geq 4$ mm and $CAL \geq 4$ mm were significantly higher in PAD patients than control subjects (Table 2). PAD patients also had significantly fewer residual teeth.

The detection of periodontopathic bacteria, CMV, C. pneumoniae and H. pylori in arterial specimens

The detection frequency of *P. gingivalis, T. denticola, A. actinomycetemcomitans* and *P. intermedia* in atherosclerostic specimens was 32%(8/25), 32%(8/25), 4%(1/25) and 20%(5/25) respectively. In 12/25 (48%) cases none of these bacteria were detected in the atherosclerotic specimens. The detection frequency of CMV and *C. pneumoniae* was 4% (1/25) and 4% (1/25), respectively. *H. pylori* was not detected in any of the specimens.

Table 3 shows the detection frequency of periodontopathic bacteria, *C. pneumoniae* and *H. pylori* in

Table 1. Demographic data of PAD patients and control subjects

	PAD patients	Control subjects
Number of subject	25	32
Age (Mean±SD)	67.6 ± 10	63.1 ± 10
Gender (Male/Female)	21/4	28/4
Heavy smoker[a]	21 (84.0%)	27 (84.4%)
Diabetes[b]	9 (36%)	3 (9.4%)

Values are given as Mean ± SD.
[a] Heavy smokers were defined as subjects who have or had a history of smoking with≥20 cigarettes per day, more than 20 years.
[b] Diabetes was defined as a history of diagnosed diabetes, use of insulin or hypoglycemic medication, a fasting blood glucose level higher than 7 mmol/l or a haemoglobin A_{1c} level above 0.07.

Table 2. Periodontal examinations in PAD patients and control subjects

	PAD patients	Control subjects	*P*
Periodontitis prevalence	68.0%	31.0%	0.004
Percentage of probing sites with PD $\geq$ 4mm(%)	14.8%	2.6%	0.003
Percentage of probing sites with CAL $\geq$ 4mm(%)	39.0%	13.4%	0.007
Number of residual teeth	13.2	24.5	<0.001

PD, probing depth; CAL, clinical attachment level.

Fontaine grade II patients and Fontaine grade III or IV patients. The higher frequency of *P. gingivalis* in Fontaine grade III or IV patients than Fontaine grade II patients (57.1% vs 22.2%) was not statistically significant ($P = 0.09$, Fisher's exact test).

Serum IgG titers against four periodontopathic bacteria

Fig. 1 shows the results of serum IgG antibody levels. The IgG titers against *P. gingivalis, T. denticola* and *P. intermedia* were significantly higher in PAD patients than control subjects. No significant difference was observed in the IgG titers against *A. actinomycetemcomitans*.

Serum inflammatory cytokine levels

In PAD patients, the presence of periodontitis was associated with a significantly increased serum IL-6 and TNF-α concentration (mean±SD, 19.60 ± 24.42 vs 3.81 ± 3.58, $p < 0.01$ and 1.41 ± 1.03 vs 0.55 ± 0.51 pg/ml, $p < 0.01$ respectively); this difference was not observed for IL-1β concentration (mean $\pm$ SD, 4.22 ± 4.05 vs 3.10 ± 3.58 pg/ml). Control subjects with periodontitis also had higher serum IL-6, TNF-α and IL-1β levels but these differences were not statistically significant (mean±SD, IL-6, 0.93 ± 0.52 vs 0.70 ± 0.52; TNF-α, 0.91 ± 0.62 vs 0.51 ± 0.51; IL-1β 0.70 ± 1.36 vs 0.35 ± 0.59 pg/ml).

Table 3. The detection frequency of periodontopathic bacteria, C. *pnemoniae* and H. *pylori* in Fontaine grade II patients and Fontaine grade III or IV patients

	Fontaine grade II patients (Intermittent claudication) $n = 18$	Fontaine grade III or IV patients (Critical limb ischemia) $n = 7$
P. *gingivalis*	4 (22.2%)	4 (57.1%)
T. *denticola*	6 (33.3%)	2 (28.6%)
A. *actino-mycetemcomitans*	0	1 (14.3%)
P. *intermedia*	3 (16.7%)	2 (28.6%)
C. *pneumoniae*	0	1 (14.3%)
H. *pylori*	0	0

No significant difference was observed between these two groups.

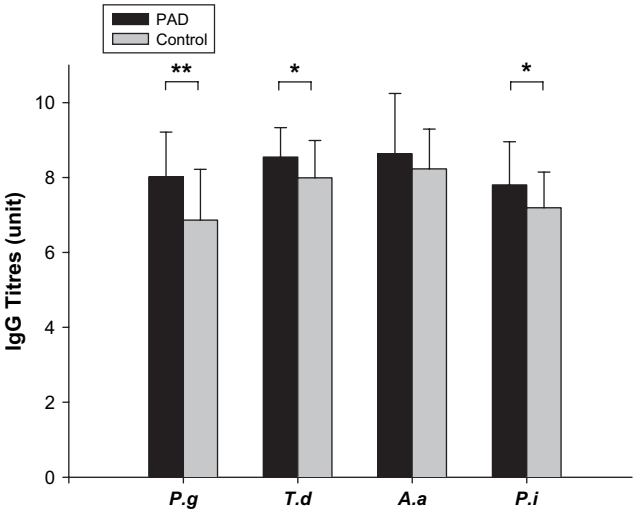

Fig. 1. Serum IgG titers against *P. gingivalis, T. denticola, A. actinomycetemcomitans* and *P. intermedia* in PAD patients and control subjects. *$P < 0.05$, **$P < 0.01$.

Association between PAD and periodontitis

Logistic regression analysis was performed in all study subjects to examine the association between PAD and periodontitis (Table 4). After adjusting for smoking, age, gender and diabetes periodontitis raised the odds ratio for having PAD to 5.45.

Discussion

This study suggests that periodontitis patients may be associated with a 5-fold increase in risk of developing PAD compared with periodontally healthy subjects. These findings support the previous epidemiological evidence of a possible etiologic relation between periodontitis and PAD.[17,18] However, our study was small and could be subject to type I errors.

Growing evidence suggests that atherosclerosis is a chronic inflammatory disease of the arteries resulting from endothelial injury, followed by macrophage migration and vascular smooth muscle proliferation.[25–27] Infectious microorganisms such as CMV, *C. pneumoniae*, *H. pylori* as well as periodontopathic bacteria have been suggested to cause endothelial dysfunction and exert inflammatory responses.[28–32] In our study, the detection frequency of CMV, *C. pneumoniae* and *H. pylori* was much lower than the detection frequency of periodontopathic bacteria.

There are several explanations for the associations between periodontitis and PAD. Locally produced pro-inflammatory mediators, such as IL-6, TNF-α and IL-1β, may spill into the circulation and exert systemic or distant effects, including the recruitment of monocytes, up-regulate endothelial adhesion

Table 4. Association of several risk factors with PAD in logistic regression model. Independent variables include periodontitis, smoking, age, gender, and diabetes

	Dependent variable: PAD	
Independent variables	Odds Ratio (95% CI)	*P* value
Periodontitis	5.45 (1.57−18.89)	0.007
Smoking	0.75 (0.13−4.43)	0.754
Age	0.99 (0.94−1.05)	0.813
Gender	1.65 (0.18−15.61)	0.661
Diabetes	0.18 (0.03−1.12)	0.065

95% CI: 95% confidence interval.

molecules, and increase macrophage uptake of lipids.[33] Preliminary observations in out study suggest that periodontitis might have contributed to the increased IL-6 and TNF-α levels in PAD patients. Loos *et al*. found higher serum IL-6 level in the peripheral blood of periodontitis patients compared with subjects without periodontitis.[7] Gorska *et al*. reported that concentrations of TNF-α and IL-1β in both serum samples and gingival tissues were significantly higher in periodontitis patients than healthy controls.[9]

Periodontopathic bacteria also may directly enhance atherogenesis. *P. gingivalis* has been demonstrated to stimulate the expression of cell adhesion molecules such as ICAM-1, VCAM-1, *P*-selectin and E-selectin in endothelial cells[31] *T. denticola* is suggested to adhere to and invade endothelial cells by secreting a specific protease.[32] In our study, PAD patients showed higher IgG titres against *P. gingivalis* and *T. denticola* and these organisms could be detected in atherosclerotic specimens to suggest that a significant proportion of PAD patients were infected by these two bacteria. Both atherosclerosis and periodontitis have been suggested to be associated with a hyperinflammatory monocyte state.[12] Monocytes in periodontitis patients are comprised significantly of activated phenotypes, which produce large amounts of IL-6.[34] Therefore, there may be other common underlying risk factors (including genetic factors) for PAD and periodontitis.

Small case-control studies, as reported here, cannot confirm causality. Periodontitis may be one of a number of inflammatory diseases associated with PAD. Further studies are necessary to determine whether periodontitis is a causal factor associated with PAD, so that more aggressive periodontal interventions are necessary.

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