High Incidence of Actinobacillus Actinomycetemcomitans Infection in Acute Coronary Syndrome

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SUMMARY

Recent epidemiological studies suggest that periodontitis is an important risk factor for coronary heart disease (CHD). The aim of this study was to evaluate the association between periodontitis and CHD, particularly acute coronary syndrome (ACS), focusing on microbiological and immunological features.

Twenty-eight CHD patients, 15 with ACS and 13 with chronic CHD, were included in this study. Coronary angiography, periodontal examination, and dental radiography were performed in all patients. Subgingival plaque, saliva, and blood samples were analyzed for the periodontopathogens Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, Treponema denticola, and Prevotella intermedia using polymerase chain reaction.

Specific serum antibody titers to the 5 periodontal pathogens were determined by enzyme-linked immunosorbent assay. It was found that 33% of the ACS patients (5/15) harbored A. actinomycetemcomitans in oral samples, whereas no A. actinomycetemcomitans (0/13) was found in the chronic CHD patients ($P < 0.05$). Furthermore, ACS patients showed significantly higher serum IgG titers to A. actinomycetemcomitans ($P < 0.05$) compared with chronic CHD. More tooth loss and alveolar bone loss were noted in ACS patients than in chronic CHD patients, although the differences were not statistically significant.

Periodontal pathogens, particularly A. actinomycetemcomitans, may play a role in the development of ACS. (Int Heart J 2007; 48: 663-675)

Key words: Acute coronary syndrome, Actinobacillus actinomycetemcomitans, Periodontopathogen

CORONARY heart disease (CHD), especially acute coronary syndrome (ACS), still represents the leading cause of adult mortality and morbidity in the world.
Coronary atherosclerosis is by far the most frequent cause of ischemic heart disease, and plaque disruption with superimposed thrombosis is the main cause of the ACS of unstable angina, myocardial infarction, and sudden coronary death.1,2) Periodontitis, a chronic inflammatory disease caused by persistent dental plaque bacteria, is characterized by the destruction of connective tissue attachment and alveolar bone and may eventually lead to tooth loss. It affects approximately 30% of the population of Western countries and 70% to 80% of the population of Japan. Several studies suggest that poor dental health and periodontal bone loss may be associated with CHD events, even after adjustment for established cardiovascular risk factors.3-5) However, other reports deny this association.6,7) Our previous investigation detected several species of periodontal bacteria in aneurysmal walls and thrombi of patients with abdominal aortic aneurysms8) or Buerger disease.9) Inflammation within the atherosclerotic plaque may lead to plaque destabilization,10) and dental plaque bacteria may also contribute to this destabilization. There are few studies that have investigated the periodontal infections in ACS and chronic CHD.

The purpose of the present study was to investigate the association between chronic periodontitis and ischemic heart disease within a Japanese population, focusing on the microbiological and immunological features of this disease and to further characterize periodontal infections in patients with ACS versus those with chronic CHD.

**Methods**

**Study population:** The study population consisted of 28 patients with CHD admitted to the Department of Cardiovascular Medicine at Tokyo Medical and Dental University. All patients were evaluated with coronary angiography. Patients with \( \geq 75\% \) coronary stenosis on angiography were defined as having CHD. The 28 CHD patients included 15 with ACS (ACS group) and 13 with stable angina pectoris or previous myocardial infarction (chronic CHD group). Patients with ACS were defined as having angina at rest and ST segment alterations with or without elevation of troponin I.

Informed consent was obtained from each subject after providing them with verbal and written explanations of the nature of the study. The study was approved by the Ethics Committee of Tokyo Medical and Dental University.

**Periodontal examination:** Periodontal examinations were performed by one dentist who was not familiar with the clinical systemic findings of these patients. Full-mouth clinical measurements including probing of pocket depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were recorded using a manual probe (PCP-UNC 15, Hu-Friedy Manufacturing Co., Chicago, IL,
USA) at 6 sites around each tooth. Oral specimens (subgingival plaque and saliva) were taken at the same time. A full-mouth set of 10 periapical radiograms was also obtained in each patient by the isometric method. Alveolar bone loss was measured on the 10 dental X-ray films according to the method of Schei, et al. The percentage of mesiodistal alveolar bone resorption was obtained for each tooth. The average of the percentage for all teeth was used as the amount of bone loss for each subject.

**Sample collection and preparation:** Blood samples were taken from all subjects during their admission to our Department of Cardiovascular Medicine. All subjects underwent a complete blood count, blood chemistry analysis, and several measures of lipid metabolism including total cholesterol (T-Chol), triglycerides (TG), and high- and low-density lipoprotein (HDL, LDL). Serum high-sensitivity C-reactive protein (CRP) and interleukin-6 (IL-6) were measured by SRL Company, Ltd. (Tokyo). Whole blood was subjected to microbiological analysis. The characteristics of the subjects are shown in Table I. Blood samples were also subjected to determine the specific serum IgG antibody responses to the periodontal pathogens tested.

Subgingival plaque and saliva samples were collected during a periodontal examination. Subgingival plaque samples were collected from the deepest pockets in each quadrant and pooled for microbiological analysis. After supragingival debridement, subgingival plaque was collected by inserting a sterile paper point (No. 30) into the pocket until resistance was felt and was kept in place for 30 sec-

<table>
<thead>
<tr>
<th>Table I. Demographic Data of the Study Population</th>
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<tbody>
<tr>
<td>ACS (n = 15)</td>
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<tr>
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</tr>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>64 ± 11</td>
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<tr>
<td>64 ± 9</td>
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<tr>
<td>0.94</td>
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<tr>
<td><strong>Hypertensive</strong></td>
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<tr>
<td>11 (73)</td>
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<td>8 (62)</td>
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CHD indicates coronary heart disease; ACS, acute coronary syndrome; LDL, low density lipoprotein; HDL, high density lipoprotein; WBC, white blood cells; Hs-CRP, high sensitivity C-reactive protein; and IL-6, interleukin-6.
onds. Paper points with plaque samples were transferred to a sterile vial. Unstimulated saliva (500 µL) was also collected from each patient in a sterile tube. All samples were stored at -80°C until analysis.

**Polymerase chain reaction (PCR) detection for periodontopathogens:** Subgingival plaque, saliva, and blood samples were subjected to a 16S rRNA-based PCR detection method\(^{12}\) to determine the presence of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, Treponema denticola, and Prevotella intermedia. The 16S rRNA-specific primers used are shown in Table II. Briefly, each 50-µL PCR reaction mixture contained 5 µL of the sample, 5 µL of 10x PCR buffer (TaKaRa, Shiga, Japan), 1.25 units of Taq DNA polymerase (TaKaRa), 0.2 mM of each deoxyribonucleotide (TaKaRa), 1.0 mM of each primer, and 1.0 mM MgCl\(_2\) for A. actinomycetemcomitans or 1.5 mM MgCl\(_2\) for T. forsythensis, P. gingivalis, and T. denticola. PCR amplification was performed in a DNA thermal cycler (PTC-200, MJ Research, Boston, MA). The temperature profile for A. actinomycetemcomitans and P. intermedia included an initial step at 95°C for 2 minutes followed by 36 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes, and a final step at 72°C for 10 minutes. The PCR temperature profile for T. forsythensis, P. gingivalis, and T. denticola included an initial step at 95°C for 2 minutes followed by 36 cycles of 95°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute, and a final step at 72°C for 2 minutes. PCR amplification products were analyzed with 1.0% agarose gel electrophoresis. The gel was stained with 0.01 mg/L ethidium bromide and photographed under ultraviolet light at 300 nm. A 100 bp DNA ladder digest (Promega, Madison, WI) served as the molecular size marker.

**Preparation of microorganisms:** A. actinomycetemcomitans ATCC 43719, P. gingivalis 381, T. forsythensis ATCC 43037, T. denticola ATCC 33520, and P. intermedia ATCC 25611 were used. A. actinomycetemcomitans ATCC 43719

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer pairs (5’ to 3’)</th>
<th>Amplicon size in bp</th>
</tr>
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<tbody>
<tr>
<td>P. gingivalis</td>
<td>ACTGGTAGGAAACTACCGAATGT</td>
<td>404</td>
</tr>
<tr>
<td>T. forsythensis</td>
<td>AGGCAGCTTTGCCATACTCGG</td>
<td>641</td>
</tr>
<tr>
<td>T. denticola</td>
<td>TGCTTACGGTGCAAGTTATACACCTGCGT</td>
<td>316</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>AAACCACATCCTGCTGAAGATTCTTCTCTCTCTCTCTCTCTCTCTCTGT</td>
<td>557</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>TCAACATCTGTATCCCTGCGT</td>
<td>575</td>
</tr>
</tbody>
</table>

PCR indicates polymerase chain reaction.
was grown in brain heart infusion (BHI) broth (Difco) supplemented with 1% (w/v) yeast extract at 37°C for 3 days in a 5% CO₂ atmosphere. P. gingivalis 381, T. forsythensis ATCC 43037, and P. intermedia ATCC 25611 were grown in brain heart infusion (BHI) broth (Difco) supplemented with vitamin K and hemin under anaerobic conditions (10% H₂, 5% CO₂ and 85% N₂) and harvested after 5 days. T. denticola ATCC 33520 was grown on New Oral Spirochete medium (NOS) under an atmosphere of 10% H₂, 10% CO₂, and 80% N₂. The microorganisms were harvested by centrifugation, washed 3 times with distilled water, and lyophilized.

Sonicated whole cell extracts of periodontopathogens were prepared according to the method of Naito and co-workers. Cells were suspended in saline (200 mg/mL, wet weight) and disrupted by sonication at 30 second intervals for a total sonication time of 3 minutes at maximum output on ice. The sonicated cell suspensions were centrifuged and the supernatants were lyophilized.

**Serum antibody titer measurement:** Specific serum IgG titers were measured by enzyme-linked immunosorbent assay as described previously using sonicated whole cell extracts of each periodontopathogen. Briefly, 96-well microtiter plates (EIA plate, Costar, Cambridge, MA) were coated with sonicated extracts (10 µg/mL) in carbonate buffer, and incubated 2 hours at 37°C. After blocking with 2% BSA in carbonate buffer, the plates were washed 3 times with PBS-T (1 × PBS, 0.05% Tween 20, pH 7.2). Serially diluted reference positive control serum (2⁴ to 2⁸, 100 µL per well) and single diluted (2¹⁰ for P. gingivalis and A. actinomycetemcomitans, 2⁸ for P. intermedia and T. denticola, and 2⁷ for T. forsythensis) patient serum were added into each well in duplicate and the plates were incubated for 2 hours at 37°C. Following incubation, the plates were washed again 3 times. Subsequently, 100 µL per well of alkaline phosphatase-conjugated goat anti-human IgG (Sigma Chemical Co., USA) was added. Following incubation, the plates were washed 3 times and developed with phosphate substrate (Sigma 104). The optical density was read using a Microplate Reader (SOFT Max®) at 405 nm with a 650 nm reference wavelength. Antibody titer was calculated according the method of Wang, et al.

**Statistical analysis:** Differences in continuous and categorical variables were examined with the unpaired t test and χ² for two group comparisons. The alpha level was set at 0.05. All statistical analyses were performed with the aid of statistical software (StatView® for Windows, Version 5.0, SAS Institute Inc., Cary, NC).

**RESULTS**

**Characteristics of the study population:** The demographic characteristics of the
study population are presented in Table I. The ACS group included 10 patients with acute myocardial infarction and 5 with unstable angina. The chronic CHD group comprised 6 patients with previous myocardial infarction and 7 with stable angina.

In the blood analyses, the levels of fibrinogen and high-sensitivity CRP were significantly higher in the ACS group than in the chronic CHD group ($P < 0.05$, $P < 0.05$, respectively, Table I). Although the levels of IL-6 in the ACS group were higher than those in the chronic CHD group, the difference did not reach statistical significance.

**Periodontal evaluation**: Subjects who had at least 1 site with a tooth pocket depth of $\geq 4$ mm and/or showed bone loss on the radiograms were considered to have periodontitis. In the present study, most subjects had periodontitis (Table II). The number of missing teeth, % of sites with pocket depth $\geq 4$ mm, % of sites with clinical attachment level $\geq 4$ mm, % of sites exhibiting BOP, and % of teeth with bone loss $\geq 25 \%$ in radiograms were also higher among patients in the ACS group than in the chronic CHD group (Table III); however, the differences were not sta-

<table>
<thead>
<tr>
<th></th>
<th>ACS ($n = 15$)</th>
<th>Chronic CHD ($n = 13$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of missing teeth</td>
<td>$10.7 \pm 2.2$</td>
<td>$8.0 \pm 1.7$</td>
<td>0.37</td>
</tr>
<tr>
<td>% of sites with PD $\geq$ 4mm</td>
<td>$16.3 \pm 5.5$</td>
<td>$8.0 \pm 4.0$</td>
<td>0.25</td>
</tr>
<tr>
<td>% of sites with CAL $\geq$ 4mm</td>
<td>$32.4 \pm 7.9$</td>
<td>$23.5 \pm 5.9$</td>
<td>0.39</td>
</tr>
<tr>
<td>Bleeding on probing, % of sites</td>
<td>$3.5 \pm 1.3$</td>
<td>$2.8 \pm 1.5$</td>
<td>0.75</td>
</tr>
<tr>
<td>Bone loss on radiographs, %</td>
<td>$30.3 \pm 5.2$</td>
<td>$23.0 \pm 3.1$</td>
<td>0.29</td>
</tr>
</tbody>
</table>

CHD indicates coronary heart disease; ACS, acute coronary syndrome; PD, pocket depth; and CAL, clinical attachment level.

**Figure 1**: A full-mouth set of 10 periapical radiograms of one subject who was diagnosed as having an acute myocardial infarction.
tistically significant. Figure 1 shows the alveolar bone resorption in one subject with an acute myocardial infarction. In this case, there was alveolar bone resorption in different areas, and 10 teeth were missing.

**Microbiological evaluation:** The results of PCR analysis of oral and blood samples for all subjects are shown in Table IV. P. gingivalis, T. forsythensis, and T.

<table>
<thead>
<tr>
<th></th>
<th>Oral samples N (%)</th>
<th>Blood samples N (%)</th>
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<tbody>
<tr>
<td></td>
<td>ACS</td>
<td>Chronic CHD</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>14 (93)</td>
<td>10 (77)</td>
</tr>
<tr>
<td>T. forsythensis</td>
<td>15 (100)</td>
<td>12 (92)</td>
</tr>
<tr>
<td>T. denticola</td>
<td>12 (80)</td>
<td>11 (85)</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>5 (33)*</td>
<td>0</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>10 (67)</td>
<td>7 (54)</td>
</tr>
</tbody>
</table>

CHD indicates coronary heart disease; ACS, acute coronary syndrome; PCR, polymerase chain reaction.

*; *P* < 0.05.

**Figure 2.** Polymerase chain reaction assay with amplified bands of periodontal bacteria-specific 16s ribosomal RNA. Lane 1, 100 bp DNA ladder; Lanes 2, 6, 10, 14, positive control; Lanes 3-4, 7-8, 11-12, and 15-16, oral samples; Lanes 5, 9, 13 and 17, blood samples (Figure 2A, subject No.2). Lane 1, 100 bp DNA ladder; Lanes 2, 5, 8, 11 and 14, positive control; Lanes 3, 6, 9, 12, and 15, oral samples; Lanes 4, 7, 10, 13 and 16, blood samples (Figure 2B, subject No.4).
denticola were most frequently detected in the oral samples from each group (Table IV). The prevalence of these 3 bacteria in the oral cavity did not differ significantly between the ACS and chronic CHD patients (93% and 77% for P. gingivalis, 100% and 92% for T. forsythensis, and 80% and 85% for T. denticola, respectively). On the other hand, A. actinomycetemcomitans was significantly highly detected from oral samples in ACS patients (33%), while no A. actinomycetemcomitans was found in the chronic CHD patients ($P < 0.05$). More ACS patients (5/15) were positive for periodontal pathogen(s) in blood samples than were chronic CHD patients (2/13), but the difference was not statistically significant (Table IV). Figure 2 shows the identification of the 5 periodontopathogens from oral and blood samples by PCR from two subjects with acute myocardial infarction.

**Serum IgG titers to periodontopathogens**: Specific serum IgG titers to the 5 periodontopathogens are shown in Figure 3. The IgG titers to A. actinomycetemcomitans were significantly higher in ACS patients than in chronic CHD patients ($P < 0.05$). No statistically significant differences were found in the IgG titers to P. gingivalis, T. forsythensis, T. denticola and P. intermedia between the ACS and chronic CHD groups.

**DISCUSSION**

Periodontitis is a multifactorial infection elicited by a complex of bacterial
species and host immune responses. Several bacterial species, such as P. gingivalis, A. actinomycetemcomitans, T. forsythensis, P. intermedia, and T. denticola have been implicated in chronic periodontitis.\textsuperscript{16}

A. actinomycetemcomitans is a gram-negative, facultatively anaerobic coccobacillus and is considered to be the major etiologic agent of localized aggressive periodontitis.\textsuperscript{17,18} It also contributes to chronic periodontitis.\textsuperscript{19,20} Furthermore, this microorganism has been isolated from several other nonoral infections, including brain abscess,\textsuperscript{21} endocarditis,\textsuperscript{22} pericarditis,\textsuperscript{23} pneumonia,\textsuperscript{24} and septicemia.\textsuperscript{25,26} Recently, Desvarieux, et al found the cumulative periodontal pathogenic burden (A. actinomycetemcomitans, P. gingivalis, T. denticola, and T. forsythensis) was significantly related with carotid artery intima thickness.\textsuperscript{27} The present study found that A. actinomycetemcomitans was detected to a significantly greater degree from oral samples of the ACS group patients than from chronic CHD patients. In addition, A. actinomycetemcomitans was also detected from the blood samples of one ACS patient who harbored A. actinomycetemcomitans in the oral cavity. The oral cavity is the likely source of systemic A. actinomycetemcomitans infections, especially in patients with periodontitis. Furthermore, ACS patients had significantly elevated serum IgG responses to A. actinomycetemcomitans compared to chronic CHD patients. Increased serum antibody to A. actinomycetemcomitans represents a destruction of the periodontal structures and a systemic challenge and dissemination of the organism leading to vascular activation. Taken together, although the sample size is small in the present study, our findings suggest that A. actinomycetemcomitans infection may play a role in the incidence of ACS. These results are supported by recent studies showing that elevated serum anti-A. actinomycetemcomitans antibody levels predict stroke\textsuperscript{28} and coronary heart disease.\textsuperscript{29}

A. actinomycetemcomitans possesses a number of putative virulence factors, including a leukotoxin that targets and destroys specific host immune cells (neutrophils and monocytes). Leukotoxin is also involved in the adhesion of A. actinomycetemcomitans.\textsuperscript{30} The organism takes advantage of its high adhesive-ness and is capable of rapid invasion and spread through eukaryotic cells.\textsuperscript{31,32}

There is currently a significant amount of evidence supporting a link between chronic inflammation in atherosclerosis and ACS. Clot formation and acute inflammatory pathways play important roles in the acceleration of local macrophage and T-cell activation, which contributes to plaque erosion or rupture. A. actinomycetemcomitans has been reported to produce an as yet unidentified superantigen that rapidly causes T-cell apoptosis.\textsuperscript{33,34} Ten, et al reported that periodontitis patients infected with A. actinomycetemcomitans harbored A. actinomycetemcomitans-specific T-cells in peripheral blood, and T-cells expressed RANK ligand (RANKL) in response to A. actinomycetemcomitans.\textsuperscript{35} RANKL
from T-cells stimulates vascular smooth muscle cells (VSMC) to produce matrix metalloproteinase-9 (MMP-9), which destabilizes atherosclerotic plaques and recently was reported to be elevated in coronary artery plaque in patients with acute myocardial infarction.

It is less clear why A. actinomycetemcomitans could not be detected in chronic CHD while there was no change in periodontal status. An elevated antibody response to A. actinomycetemcomitans has been reported to function to eliminate the bacteria. A. actinomycetemcomitans may be eliminated by a high level of antibody to A. actinomycetemcomitans in ACS. Subsequently, A. actinomycetemcomitans could not be detected in the chronic period. The presence of P. gingivalis and T. denticola, even though after the eradication of A. actinomycetemcomitans, may have sustained the periodontal status.

P. gingivalis, T. denticola, and T. forsythensis were detected at high levels in all subjects in the present study (Table IV). These 3 microorganisms have been classified as the “red complex,” which has been associated with chronic periodontitis. Within this group, P. gingivalis and T. denticola are frequently found together in diseased sites. Kurihara, et al recently reported that P. gingivalis and T. denticola were the most frequently detected periodontopathogens in abdominal aortic aneurysms. In contrast, a study by Marques, et al has shown that among periodontal pathogens, A. actinomycetemcomitans was found in excised aortic aneurysms by using multiplex PCR, while other organisms from the red complex were not detected. In the present study, slightly more T. denticola DNA was detected from blood of ACS patients than from chronic CHD patients. T. denticola may be also associated with the development of ACS. However, the serum anti-T. denticola IgG titers were not different between the two groups; this issue requires further study using more patients.

Associations between tooth loss and CHD and atherosclerosis endpoints have been reported. Periodontal disease represents the main cause of tooth extraction after the age of 40. In the present study, more tooth and alveolar bone loss, and worse periodontal status (Table II) were noted in ACS patients than in chronic CHD patients and the difference was not significant, whereas increased serum antibody to A. actinomycetemcomitans was associated with ACS compared with chronic CHD. These findings suggest that the host immune and/or inflammatory response to periodontal pathogens may be a more direct measure than the clinical and radiographic measures for the association between periodontal disease and ACS. Additional studies are necessary to better define the relationship between periodontal conditions and ACS.

In conclusion, periodontal pathogens, particularly A. actinomycetemcomitans, may have played an important role in the development of ACS in this Japanese population. We believe that prospective randomized studies with large
numbers of patients are needed to further determine the risk of periodontal infections, especially A. actinomycetemcomitans infection, for acute coronary syndrome. In the future, this pathogen may represent a therapeutic target in the prevention of coronary heart disease.

ACKNOWLEDGMENTS

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REFERENCES
