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Identification of periodontopathic bacteria in gingival tissue of Japanese periodontitis patients

K. Thiha[1,2], Y. Takeuchi[1,2], M. Umeda[1,2], Y. Huang[1,2], M. Ohnishi[1,2], I. Ishikawa[3,4]

[1]Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, [2]Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, [3]Professor Emeritus, Tokyo Medical and Dental University, [4]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

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Introduction: The identification of invading periodontopathic bacteria in tissues is important to determine their role in the pathogenesis of periodontal disease. The objective of this study was to identify periodontopathic bacteria in diseased gingival tissue of periodontitis patients.

Methods: Subgingival plaque and gingival tissue were collected from 32 generalized chronic periodontitis (CP), 16 generalized aggressive periodontitis (GAgP) and eight localized aggressive periodontitis (LAgP) patients. Detection frequencies and quantities of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythensis* were investigated by polymerase chain reaction. The prevalences of *Streptococcus oralis* and *Streptococcus sobrinus* were also examined and the distribution of *A. actinomycetemcomitans* serotypes was observed.

Results: *P. gingivalis and T. forsythensis* were detected in approximately 70% of tissue samples and 50% of plaque samples in the three periodontitis groups. Prevalence of *A. actinomycetemcomitans* in tissue samples was higher in the LAgP (63%) group than in either the CP (16%) or the GAgP (38%) group. *A. actinomycetemcomitans* serotype c was detected in 50% of LAgP patients. Detection frequencies of *S. oralis* and *S. sobrinus* were markedly low in both plaque and tissue samples from all three periodontitis groups. Amounts of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in the tissue samples were not different among the three periodontitis groups.

Conclusion: *P. gingivalis*, *A. actinomycetemcomitans and T. forsythensis* can localize in diseased gingival tissue and may be involved in periodontal tissue destruction. Serotype c is the predominant serotype of *A. actinomycetemcomitans* in Japanese LAgP patients.

Key words: *Actinobacillus actinomycetemcomitans* serotype; gingival tissue; periodontopathic bacteria

Makoto Umeda, Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8549, Japan
Tel.: +81 3 5803 5488;
fax: +81 3 5803 0196;
e-mail: umeda.peri@tmd.ac.jp
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Over 500 bacterial species are capable of colonizing the oral cavity (37). Among these, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythensis* (formerly *Bacteroides forsythus*) are described as the major pathogens associated with periodontal breakdown (59). Subgingival biofilms containing these bacteria cause an inflammation in the tooth-supporting tissue and lead to a progressive loss of periodontal ligament and alveolar bone (10).

The presence of oral bacteria in periodontal tissues has been reported in patients with advanced periodontitis (7, 11, 31, 39). Some histological studies have reported the presence of *P. gingivalis* and *A. actinomycetemcomitans* in the gingival tissues of periodontitis patients (12, 34, 40), and higher numbers of these bacteria were found in active destructive periodontal lesions than in non-active sites (41). In a recent study using the fluorescent *in situ* hybridization technique with confocal microscopy, intercellular *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* were found in human buccal epithelial cells (38). Attachment and penetration of these bacteria to oral epithelial cells were also demonstrated by *in vitro* model studies (2, 16, 44). These bacteria seem to have the ability to persist against immune mechanisms and can remain in host tissues (34).

The invasion of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* into gingival tissue consolidates the infection and also affects the periodontal conditions.

Not only the direct effects of the virulence factors of bacteria, but also the considerable amount of bacterial invasion into periodontal tissues would lead to severe inflammatory responses in the host following periodontal tissue destruction. The load of tissue-invading bacteria seems to be reflected in the tissue-destructive form taken by the periodontitis. However, the specific relationship of these three periodontopathic bacteria with the tissue destruction found in the various clinical forms of periodontitis is still obscure.

Periodontopathic bacterial DNAs, including those of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* have been detected in atheromatous plaques (17, 24) and recently, these bacteria have become a focus as risk factors for vascular diseases (21, 25) and stroke (13). Bacteria that invade the highly vascularized gingiva can spread through the bloodstream (23, 26), and this phenomenon may relate to these systemic diseases. Identification of the invading bacteria in periodontal tissues is necessary to determine their role as risk factors in systemic diseases, as well as in the pathogenesis of periodontitis, and to evaluate how their presence affects diagnostic and therapeutic procedures.

The objective of this study was to identify the periodontopathic bacteria in diseased gingival tissue from Japanese patients with different forms of periodontitis. Detection of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in plaque samples and the quantity of bacteria in tissue samples, were investigated by conventional and real-time polymerase chain reactions (PCR). The distribution of *A. actinomycetemcomitans* serotypes in tissue samples was also examined.

Materials and methods

Subjects

Periodontitis patients visiting the Tokyo Medical and Dental University were selected for this study. The subjects included 34 with generalized chronic periodontitis (CP), 17 with generalized aggressive periodontitis (GAgP) and eight with localized aggressive periodontitis (LAgP), all of whom were diagnosed based on the classification described by the American Academy of Periodontology in 1999 (1). No patient had any relevant systemic diseases and none had taken any antibiotics within the previous 3 months. No subject currently smoked and all were in good general health. The study protocol was approved by the Ethical Committee of our institution, and informed consent was obtained from each patient. All patients had received initial periodontal treatment consisting of scaling and root planing and had then been given oral hygiene instruction by competent periodontists.

Sampling procedures for subgingival plaque and gingival tissues

Three weeks after scaling and root planing, clinical parameters, including probing pocket depth, clinical attachment level and bleeding on probing, were measured and the site with the deepest probing pocket depth was chosen as the sampling site. A subgingival plaque sample was taken with a sterile paper point as in the previous report by Takeuchi et al. (50). The paper point was placed into a 1.5-ml microtube containing 500 μl sterile distilled water. After the gingival flap had been made, diseased gingival tissue was taken by sterilized curette at the bone defect site corresponding to the subgingival plaque sampling site. A total of 40–55 mg (mean 50 mg) of gingival tissue was collected from each patient.

Bacterial detection and quantification in clinical samples

The paper point sample was mixed for 1 min by vortexing and a plaque suspension was prepared. A collected tissue sample was separately washed twice with physiological saline. To examine the contamination during tissue sampling 500 μl of the latter wash solution was taken for examination. Washed tissue sample was minced into small fragments and homogenized manually using a mortar and pestle. Then, the sample was transferred into 500 μl of new sterilized saline. Bacterial DNA was extracted using a High Pure PCR Template Preparation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and stored in −20°C until the PCR procedure was performed.

The presence of bacterial DNA for *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in plaque, tissues samples and wash solution from each patient was examined by conventional PCR. The presence of *Streptococcus oralis* and *Streptococcus sobrinus* in periodontitis was also examined. *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 43718 and *T. forsythensis* ATCC 43037 were cultivated, and the numbers of bacterial cells were determined using a Petroff–Hauser counting chamber as described by Umeda et al. (52). *S. oralis* OMZ 607 and *S. sobrinus* OMZ 176 were anaerobically cultivated for 3 days on trypticase soy blood agar plates (49) and the cells were harvested. The DNAs of these bacterial strains were extracted and used as a positive control in the present study. The primer sequences are described in Table 1. Conventional PCR procedures were manipulated as reported in Ashimoto et al. (3) and Hoshino et al. (19).

P. gingivalis, *A. actinomycetemcomitans* and *T. forsythensis* in tissue samples were quantified using real-time PCR. LightCycler II (Roche Diagnostic, Mannheim, Germany) and double-stranded DNA binding dye SYBR Green I were utilized in this experiment. PCR was performed as previously reported by Sakamoto et al. (43). Briefly, amplification was performed in a 20 μl reaction mixture containing 2 μl

Table 1. PCR primers used in this study

Target	Sequence (5′ → 3′)	Product size (bp)	Source
P. gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404	(45)
A. actinomycetemcomitans	ATG CCA ACT TGA CGT TAA AT AAA CCC ATC TCT GAG TTC TTC TTC	557	(3)
T. forsythensis	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641	(45)
S. oralis	TCCCGGTCAGCAAACTCCAGCC GCAACCTTTGGATTTGCAAC	374	(19)
S. sobrinus	GATGATTTGGCTCAGGATCAATCCTC ACTGAGCCAGTAGTAGACTTGGCAACT	328	(19)
A. actinomycetemcomitans			
Serotype a	GCAATGATGTATTGTCTTCTTTTGGA CTTCAGTTGAATGGGGATTGACTAAAAC	428	(48)
Serotype b	CGGAAATGGAATGCTTGC CTGAGGAAGCCTAGCAAT	298	(48)
Serotype c	AATGACTGCTGTCGGAGT CGCTGAAGGTAATGTCAG	559	(48)
Serotype d	TTACCAGGTGTCTAGTCGGA GGCTCCTGACAACATTGGAT	690	(48)
Serotype e	CGTAAGCAGAAGAATAGTAAACGT AATAACGATGGCACATCAGACTTT	211	(48)

template DNA, 2 μl LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals), 1 μM of each primer, and either 4 mM $MgCl_2$ for *P. gingivalis* and *T. forsythensis* or 3 mM $MgCl_2$ for *A. actinomycetemcomitans*. The initial denaturation step at 95°C for 2 min was followed by 40 cycles that comprised heating at 95°C for 20 s and then annealing steps were 60°C in 5 s for *P. gingivalis*, 55°C in 5 s for *A. actinomycetemcomitans* and 62°C in 5 s for *T. forsythensis*. Heating was continued at 72°C for 16 s for *P. gingivalis*, 22 s for *A. actinomycetemcomitans* and 26 s for *T. forsythensis* and a last step was performed of 1 s at 84°C for *P. gingivalis* and *A. actinomycetemcomitans*, and at 83°C for *T. forsythensis*. Fluorescent products were detected at the last step of each cycle and melting curves were used to determine the specificity of the PCR. Quantification of clinical samples was calculated using LightCycler Data Analysis software based upon the known cell counts of the control bacterial strain. The log-linear portion of the standard amplification curve was identified and the crossing point was the intersection of the best-fit line through the log-linear region and the noise band. The standard curve was the plot of the crossing point against the log of the cell numbers. Bacterial cell numbers per ml were calculated, and the minimum detection level of the real-time PCR assay was 10^3 cells/ml in sample suspension.

A. actinomycetemcomitans serotype-specific genotyping

A. actinomycetemcomitans serotypes were examined by PCR using a DNA thermal cycler (PTC-200; MJ Research, Boston, MA). Primer designs (Table 1) and PCR protocol were performed as described by Suzuki et al. (48) with some modifications. Briefly, 1 μM of each of the indicated primers and 2 μl template DNA in a 10 μl PCR mixture containing 2.5 units of *TaKaRa Ex Taq*™ polymerase (TAKARA Bio Inc., Shiga, Japan), 2 μl 10× *Ex Taq*™ buffer with $MgCl_2$, 2.5 mM each of dNTPs and PCR-grade water. After denaturation at 96°C for 2 min, a total of 36 cycles was performed; each cycle comprising 15 s of denaturation at 94°C, followed by 30 s of annealing at 56°C for serotype a, 54°C for serotypes b, c and e, and 57°C for serotype d, respectively. The final step was a 60 s extension at 72°C. DNA from *A. actinomycetemcomitans* ATCC 29523 (serotype a), ATCC 43718 (serotype b), NCTC 9710 (serotype c), IDH 781 (serotype d) and OMZ 534 (serotype e) were employed as positive controls, and the specificity of the primers was also confirmed (Fig. 1). PCR products were analysed by 1.0% agarose gel electrophoresis using 0.01 μg/ml ethidium bromide, and the product band was observed under 300 nm ultraviolet light (50).

Statistical analysis

All data were handled in the StatView software package. The difference of bacterial prevalence among CP, GAgP and LAgP groups were tested by Fisher's exact test. The prevalence of bacteria between plaque samples and tissue samples was also examined. Quantity of bacterial DNA was transformed as a log number because of the skewed distribution among the subjects. One-way factorial analysis of variance was applied to compare probing pocket depth, clinical attachment level and quantity of each bacterial DNA among the three periodontitis groups. All statistical tests were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

All the plaque, tissue and wash solution samples from each patient were investigated by conventional PCR assay to detect bacteria. If bacterial DNA was detected in the wash solution, the corresponding tissue sample would be considered as having been bacterially contaminated during the sampling procedures, and consequently dropped from data collection. With this taken into account, two subjects from the CP group and one from the GAgP group were excluded and the final participants were a total of 56 subjects consisting of 32 CP patients, 16 GAgP patients and eight LAgP patients. Clinical data of the sampling sites are summarized in Table 2. No statistically significant difference was observed in the mean values of probing pocket depth, clinical attachment level and the percentage of sites that were positive for bleeding on probing among the three periodontitis groups.

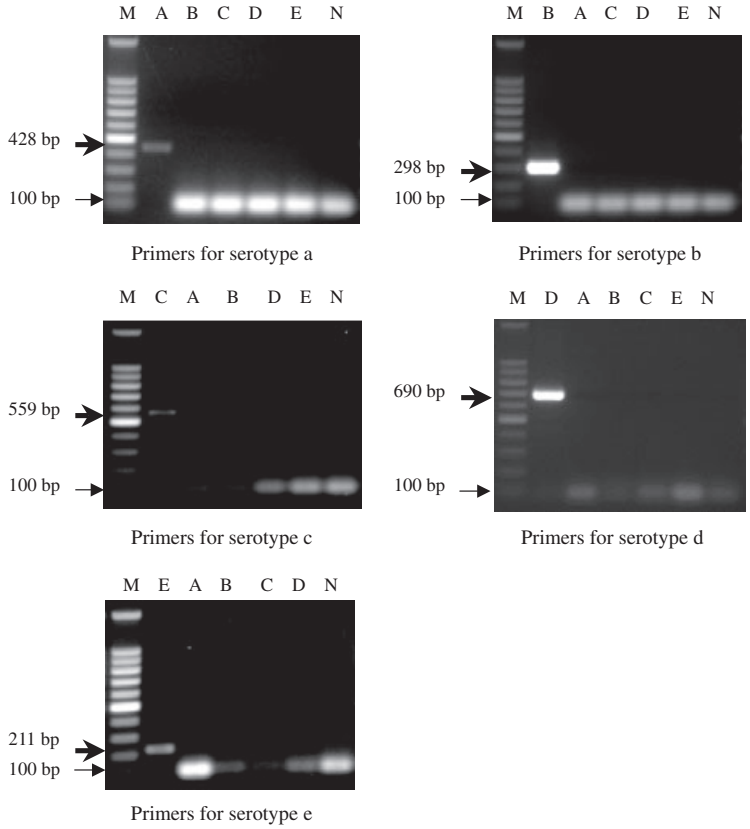

Fig. 1. PCR analysis for *Actinobacillus actinomycetemcomitans* serotype-specific DNA. Lane M, molecular size marker (100-bp DNA ladder); lane A, *A. actinomycetemcomitans* ATCC 29523 (serotype a); lane B, *A. actinomycetemcomitans* ATCC 43718 (serotype b); lane C, *A. actinomycetemcomitans* (NCTC 9710) serotype c; lane D, *A. actinomycetemcomitans* IDH 781 (serotype d); lane E, *A. actinomycetemcomitans* OMZ 534 (serotype e) and lane N, negative control. Large arrow indicates a single DNA band of the predicted size obtained by PCR. Small arrow indicates 100 bp.

Table 2. Clinical data

	CP	GAgP	LAgP
Number of subjects	32	16	8
Gender (male : female)	12 : 20	9 : 7	4 : 4
Age (mean ± 1SD)	55.13 ± 7.46	35.07 ± 8.23	31.29 ± 5.56
Sampling sites			
PPD (mean ± 1SD; mm)	5.74 ± 1.34	6.10 ± 1.46	6.37 ± 2.04
CAL (mean ± 1SD; mm)	6.26 ± 1.73	6.39 ± 2.06	6.74 ± 2.27
BOP (% of positive sites)	67	69	86

CP, chronic periodontitis; GAgP, generalized aggressive periodontitis; LAgP, localized aggressive periodontitis; PPD, probing pocket depth; CAL, clinical attachment level; BOP, bleeding on probing.

The detection frequencies of bacteria in plaque and tissue samples are summarized in Table 3. High detection rates of *P. gingivalis* and *T. forsythensis* were observed in gingival tissue of the three periodontitis groups. The prevalence of *P. gingivalis* and *T. forsythensis* was not significantly different in the CP, GAgP and LAgP patients in either the plaque or the tissue samples. The detection frequency of *A. actinomycetemcomitans* in the tissue sample was higher in LAgP patients than in the GAgP and CP patients, although a statistical difference was only found between the LAgP and CP groups ($P < 0.05$). Detection frequencies of *P. gingivalis* and *T. forsythensis* in tissue samples were higher than in plaque samples, but the difference was not significant. A similar tendency was also observed in the prevalence of *A. actinomycetemcomitans* in LAgP patients. Only a few tissue and plaque samples were positive for *S. oralis* and *S. sobrinus* in three periodontitis groups, and their detection frequencies were lower than those of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis.*

Figure 2 shows the amounts of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in bacterium-positive tissue samples. Amount of *P. gingivalis* was higher than *A. actinomycetemcomitans* and *T. forsythensis* in the tissue samples of all periodontitis groups. The amounts of each bacterium were not markedly different among the CP, GAgP and LAgP patients in this experiment. The distribution of *A. actinomycetemcomitans* serotypes in tissue samples is shown in Table 4. *A. actinomycetemcomitans* serotype c was commonly found in *A. actinomycetemcomitans*-positive samples of all the periodontitis groups and it was detected in 50% of LAgP patients. One sample taken from the GAgP group was not classified as any serotype. Multiple serotypes were simultaneously found in a few samples. Three serotypes (serotype b, c and d) and two serotypes (serotype a and d) were identified in two samples from the GAgP group. Two serotypes (serotype b and c) were detected in one sample from the LAgP group.

Discussion

In the present study, we collected the gingival tissue 3 weeks after scaling and root planing and examined the presence of the DNA of three periodontopathic bacteria. It is known that transient bacteremia can occur after scaling and root planing (53), and this phenomenon suggests that bacteria can be driven into the gingival epithelium by the periodontal instrumentation and then spread through the bloodstream. However, it is also reported that such bacteremia could last only several minutes (29), and that those bacteria driven inside the hosts seem to be quickly trapped and efficiently degraded by the host immune systems (23). Moreover, gingival epithelial cells generally turn over within 5–7 days (26), and the remaining bacterial DNA in the epithelial layers may be eliminated. We considered that the bacterial DNA driven inside by the periodontal instrumentation might have been cleared before sampling.

Approximately 70% of tissue samples were positive for *P. gingivalis* and *T. forsythensis* in GAgP and LAgP patients, showing that their prevalence was as high as that in the CP group. Our findings indicate that these two bacteria can be harbored in diseased gingival tissues and may be associated with perio-

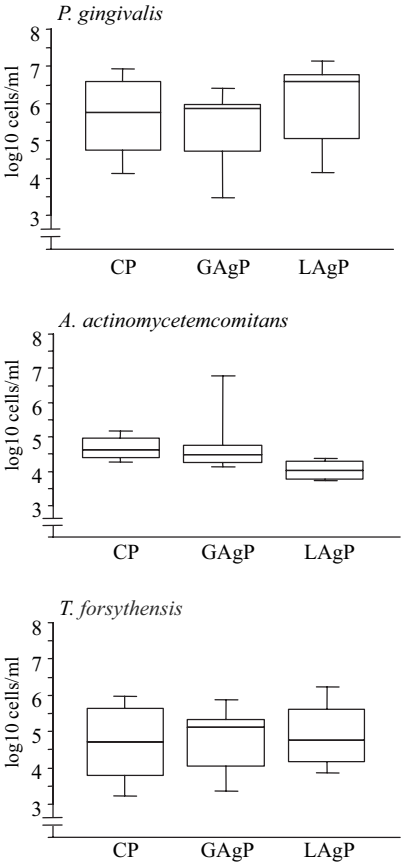

Fig. 2. Quantitative analysis in tissue samples was made by real-time PCR. Statistical comparison (log-transformed number of cell counts) was performed for each bacterium among CP, GAgP and LAgP patients. The box represents the first and third quartiles (rectangular boxes); the line within the box is the median.

Table 3. Prevalence of bacteria in plaque and tissue samples

	CP	GAgP	LAgP
P. gingivalis			
Plaque	23/32 (72)	8/16 (50)	5/8 (63)
Tissue	25/32 (78)	11/16 (69)	7/8 (88)
A. actinomycetemcomitans			
Plaque	6/32 (19)	4/16 (25)	3/8 (38)
Tissue	5/32 (16)*	6/16 (38)	5/8 (63)*
T. forsythensis			
Plaque	22/32 (69)	7/16 (44)	5/8 (63)
Tissue	24/32 (75)	13/16 (81)	6/8 (75)
S. oralis			
Plaque	2/32 (6)	1/16 (6)	0/8 (0)
Tissue	3/32 (9)	2/16 (13)	0/8 (0)
S. sobrinus			
Plaque	0/32 (0)	0/16 (0)	0/8 (0)
Tissue	0/32 (0)	0/16 (0)	0/8 (0)

Data are given as bacterium-positive sample/total samples (%).
CP, chronic periodontitis; GAgP, generalized aggressive periodontitis; LAgP, localized aggressive periodontitis.
*$P < 0.05$.

Table 4. Distribution of *Actinobacillus actinomycetemcomitans* serotypes in tissue samples

	Number of samples		
	CP	GAgP	LAgP
A. actinomycetemcomitans (+)/total	5/32	6/16	5/8
A. actinomycetemcomitans			
Serotype a	1	1	1
Serotype b	1	1	1
Serotype c	3	4	4
Serotype d	0	2	0
Serotype e	0	0	0
Non-typeable	0	1	0

CP, chronic periodontitis; GAgP, generalized aggressive periodontitis; LAgP, localized aggressive periodontitis.

dontal tissue destruction. These two species are obligate anaerobic bacteria, and their characteristics might be favorable to them residing in the tissues that provide a low-oxygen environment. Several studies have also described how *P. gingivalis* and *T. forsythensis* were frequently detected in the subgingival plaque of patients with aggressive periodontitis (8, 20, 49, 57). Taken together, these reports demonstrate the role of *P. gingivalis* and *T. forsythensis* in the tissue destruction of aggressive periodontitis.

A. actinomycetemcomitans is known as one of the pathogens most responsible for LAgP (5, 60). Christersson et al. (6) have shown that substantial suppression of subgingival *A. actinomycetemcomitans* was not achievable by scaling and root planing alone, but could be accomplished by surgical removal of periodontal tissue in LAgP patients. The present findings showed that over half of LAgP patients harbored *A. actinomycetemcomitans* in tissue samples, and its prevalence was relatively higher than in subgingival plaques. It is considered that its invading ability would allow *A. actinomycetemcomitans* to evade the mechanical debridement. Localization of *A. actinomycetemcomitans* in the periodontal tissue of LAgP patients may cause severe tissue destruction. Gwinn et al. (14) demonstrated that 29 of 30 LAgP patients had a defect in their neutrophils and decreased chemotactic activity. Although the exact mechanism has not yet been explained, this apparent major defect in the neutrophils seemed more susceptible to *A. actinomycetemcomitans* than other bacteria (18).

The detection frequencies of periodontopathic bacteria in tissue samples were generally higher than those in plaque samples. The most likely reason for this phenomenon is the collection of the subgingival plaque and tissues samples after initial periodontal treatment. A certain amount of periodontopathic bacteria would be eliminated from the periodontal pockets by the initial treatment, whereas some bacteria that were already residing in the tissues could still remain. Since there would be relatively more bacterial DNA in the tissue samples than in the plaque samples, the detection frequencies might be increased in the tissue samples.

To further our understanding of the bacterial presence in periodontal tissue, we also examined the presence of *S. oralis* and *S. sobrinus* in the same samples. *S. oralis* is known as the predominant colonizer in the early stage of dental plaque biofilm formation (27), while *S. sobrinus* is directly involved in the formation of human dental caries (28). These two bacteria seem less pathogenic for periodontitis, and our results also showed that their prevalences were markedly low in both plaque and tissue samples of periodontitis patients. In previous reports, *S. oralis* was detected in approximately 50% (54), while *S. sobrinus* was rarely found (33), in gingival plaque samples of periodontitis patients. Although there is little information about the invasion of these two bacteria in periodontal tissues, Colombo et al. (7) used DNA probes and showed that *S. oralis* was detected in 34% of crevicular epithelial cell samples from periodontitis patients. We assumed that the considerable amount of *S. oralis* could be eliminated from the periodontal pockets by initial treatments, and its prevalence in subgingival plaque would decrease in the present study. Furthermore, the possibility of invasion might become low and it could lead to the low prevalence of this bacterium in gingival tissue.

Socransky et al. (46) reported that there must be a sufficient number of pathogenic bacteria for the onset of disease. It has been suggested that there is a positive relationship between the severity of periodontal tissue destruction and the quantities of some periodontopathic bacteria in subgingival plaques (15, 47). In our results, *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* were observed in the tissue samples, but the amounts of each bacterium did not differ among CP, GAgP and LAgP patients. In the present study, tissue samples were collected from the corresponding sites of deep periodontal pockets, which still remained after initial periodontal treatment. The clinical periodontal conditions were similarly severe in the sampling sites of the patients from different periodontitis groups. This may be the reason that differences in the amount of bacteria were not observed among the three periodontitis groups.

In contrast, the amount of *P. gingivalis* in tissue samples appeared higher compared with that of *A. actinomycetemcomitans* and *T. forsythensis* in all three groups. Some previous reports showed that the quantity of *P. gingivalis* was higher than that of *A. actinomycetemcomitans* and *T. forsythensis* in subgingival plaque from periodontitis patients (49, 54). The abundance of *P. gingivalis* in subgingival plaque favored the frequent opportunity for bacterial invasion and resulted in increased numbers of this bacterium in gingival tissue. Moreover, *in vitro* study suggested that *P. gingivalis* could multiply in human oral epithelial cells (30). However, it should be borne in mind that PCR actually detects specific DNA fragments, and it is possible that DNA from dead bacteria in tissue might also be detected. We considered that although the volume of such DNA fragments in gingival tissue was limited, it could increase the number of bacterial cells in the present study.

The implication of *A. actinomycetemcomitans* in LAgP was confirmed by the fact that the prevalence of this bacterium was found to differ significantly between tissue samples from CP and LAgP patients. However, the level of *A. actinomycetemcomitans* in the tissue samples was not statistically different among the three periodontitis groups. We assumed that various clinical forms of periodontitis might be associated with the different pathogenic potentials of *A. actinomycetemcomitans* strains and focused on its serotypes. *A. actinomycetemcomitans* is classified into six serotypes (a–f) based on the serological specificity of polysaccharide antigens which exit on the surface of this bacterium (22, 58). It has been suggested that some *A. actinomycetemcomitans* serotypes are more closely associated with periodontal disease than others (59). Some studies reported that *A. actinomycetemcomitans* strains Y4 and JP2 (serotype b) had potent pathogenicity (4, 35) and were strongly associated with LAgP patients from the United States (61). *A. actinomycetemcomitans* serotype b has

been indicated as a highly pathogenic serotype. In this study, we performed *A. actinomycetemcomitans* serotype-specific genotyping by PCR instead of using immunological procedures. This PCR method seems to be sensitive and it is easy to identify serotypes of *A. actinomycetemcomitans* in clinical samples contaminated with various other bacteria (48, 58). Our results showed that *A. actinomycetemcomitans* serotype c was predominantly identified in the gingival tissues of Japanese LAgP patients, while the prevalence of serotype b was rather low. A similar tendency was observed in *A. actinomycetemcomitans*-positive tissue samples from GAgP and CP patients. Several studies have shown geographical discrepancies in the distribution of *A. actinomycetemcomitans* serotypes (9, 51, 56), and our results do not rule out this possibility. There are previous reports of serotype c being commonly found in the subgingival plaque of *A. actinomycetemcomitans*-positive subjects from some Asian populations (32, 42, 55, 58).

One strain that could not be typed was detected from sixteen *A. actinomycetemcomitans*-positive samples. It is still unclear whether this non-typeable strain had a serotype f-specific genotype or a different one. Paju et al. (36) indicated that non-serotypeable *A. actinomycetemcomitans* isolates might originate from serotypeable isolates. Further investigation is needed to clarify the characteristics of this non-typeable strain. In the present study, multiple serotypes of *A. actinomycetemcomitans* were detected in a few samples. It has been suggested that patients are usually infected by only a single serotype of *A. actinomycetemcomitans* (61). Suzuki et al. (48) investigated the subgingival plaque samples of five periodontitis patients using *A. actinomycetemcomitans* serotype-specific genotyping and also found only a single serotype in each sample. In contrast, several studies have reported multiple serotypes of *A. actinomycetemcomitans* in the same sample from periodontitis patients (32, 51, 56, 58). Although results were inconsistent, it is possible that periodontitis patients might be infected by multiple serotypes of this bacterium.

In conclusion, the localization of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* was observed in diseased gingival tissues of various clinical forms of periodontitis. In Japanese periodontitis patients, the role of *P. gingivalis* and *T. forsythensis* in LAgP and GAgP is as important as in CP for periodontal tissue destruction. Serotype c is the predominant serotype of *A. actinomycetemcomitans* in LAgP patients.

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