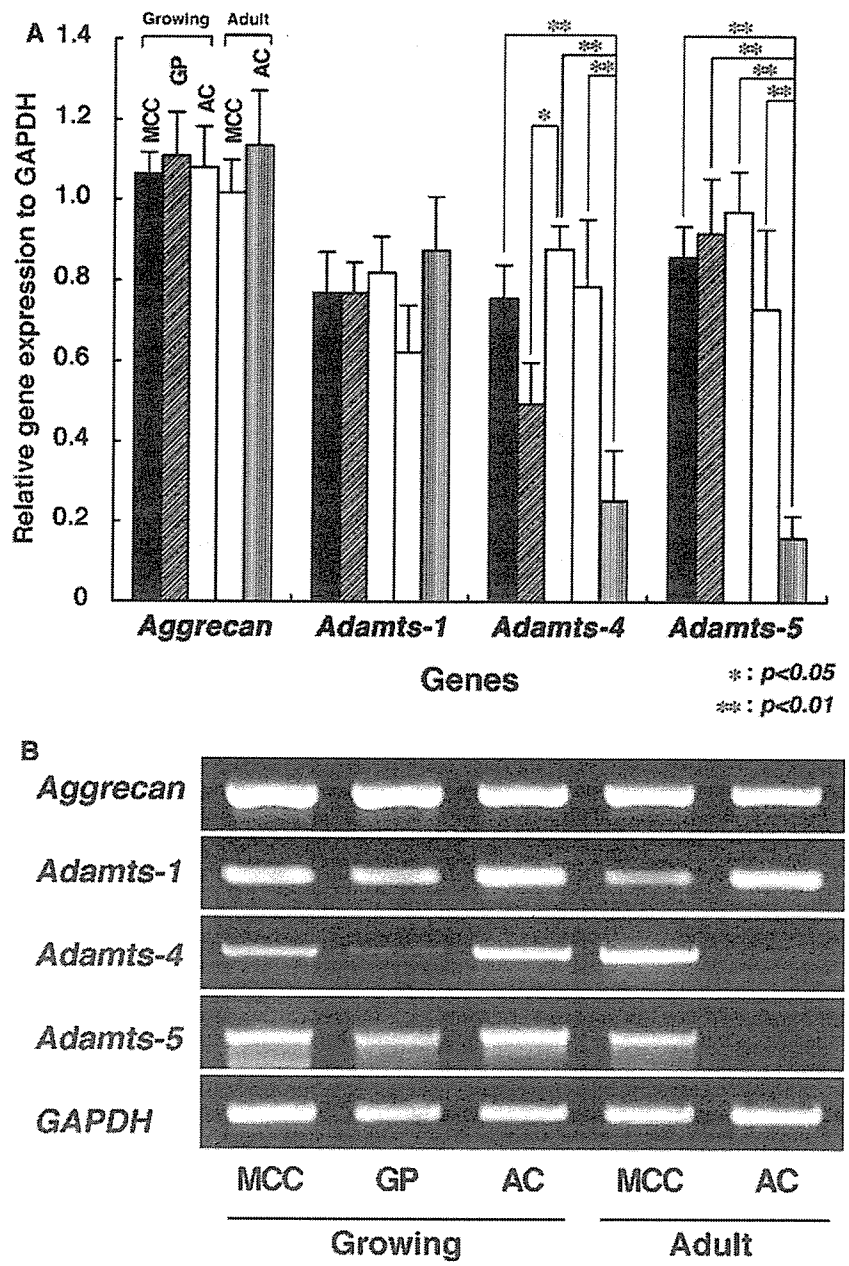

Fig. 7 Graph indicating the results of semiquantitative RT-PCR (**a**) and representative images of agarose gel electrophoresis (**b**) ($n=3$). *MCC* mandibular condylar cartilage (*closed bar* growing; *open bar* adult); *GP* growth plate cartilage (*oblique stripe bar*); and *AC* articular cartilage (*shaded bar* growing; *vertical stripe bar* adult). $*P<0.05$ and $**P<0.01$

disruption of active ADAMTS-5, but not ADAMTS-4, inhibits experimentally induced inflammatory degeneration of cartilage (Glasson et al. 2005; Stanton et al. 2005) in growing mice. Therefore, it may be that ADAMTS-5 is a major aggrecanolytic enzyme contributing not only to such pathological processes, but also to physiological degradation of ECM molecules during the growth period.

After growth, all ADAMTSs expressed in the mature chondrocytes of condylar cartilage may play a role in the physiological turnover of aggrecan in order to maintain cartilage tissue. However, ADAMTS-1 and ADAMTS-4, but not ADAMTS-5, could contribute to the physiological turnover of aggrecan in aged articular cartilage. Therefore, ECM remodeling in aged mandibular condylar cartilage could be regulated differently from that in articular cartilage.

In summary, ADAMTS-5 appears to contribute mainly to degradation of ECM molecules such as aggrecan in growth plate and condylar cartilage, depending upon its ECM composition and cellular organization during growth. In conclusion, the results of the present study reveal that ECM metabolism by ADAMTSs and expression of ADAMTSs in primary and secondary cartilage may be differentially regulated during growth and aging, depending upon the functional differences in different types of cartilage.

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Profiling of bacterial flora in crevices around titanium orthodontic anchor plates

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Key words: bacteria, bone screws, orthodontic appliances, polymerase chain reaction, 16S ribosomal RNA

Abstract

Objectives: The aims of this study were to characterize the microflora in crevices around titanium orthodontic anchor plates using anaerobic culture and molecular biological techniques for bacterial identification, and to compare the microbial composition between crevices around anchor plates and gingival crevices.

Material and methods: Samples from crevices around titanium anchor plates and healthy gingival crevices of 17 subjects (aged 20–29) were cultured anaerobically, and isolated bacteria were identified by 16S rRNA sequencing.

Results: The average logarithm colony-forming units/ml were 6.84, 7.51 and 8.88 in healthy anchor plate crevices, inflamed anchor plate crevices and healthy gingival crevices, respectively, indicating that the bacterial density of anchor plate crevices was lower than that of healthy gingival crevices. Of 184 strains isolated from healthy anchor plate crevices of seven subjects, 108 (59%) were anaerobic bacteria, while 73 (40%) were facultative bacteria. Predominant isolates were Gram-negative rods, such as *Campylobacter* (12%), *Fusobacterium* (10%) and *Selenomonas* (10%), and Gram-positive facultative bacteria, such as *Actinomyces* (17%) and *Streptococcus* (8.2%). Of 133 strains isolated from inflamed anchor plate crevices of three subjects, 110 (83%) were anaerobic bacteria, while predominant isolates were Gram-negative rods, such as *Prevotella* (47%), *Fusobacterium* (33%) and *Campylobacter* (16%). On the other hand, of 146 strains isolated from healthy gingival crevices of seven subjects, 98 (67%) were facultative bacteria, while 45 (31%) were anaerobic bacteria. Predominant isolates were Gram-positive facultative bacteria, such as *Actinomyces* (37%) and *Streptococcus* (20%).

Conclusions: These results suggest that the environment in crevices around titanium orthodontic anchor plates is anaerobic and supportive of anaerobic growth of bacteria, which may trigger inflammation in the tissue around the plates.

Microbial flora at the interface between histocompatible artificial material and mucosal epithelium is one of the most important factors for the prognosis of dental implants (Mombelli et al. 1987; Rosenberg et al. 1991; van Winkelhoff et al. 2000). It has been reported that Gram-negative obligate anaerobes predominantly comprised the bacterial flora in peri-implantitis pockets as well as periodontal pockets (Mombelli et al. 1987), whereas Gram-positive facultative anaerobes are predominant in healthy peri-implant crevices of successful implants (Mombelli & Mericske-Stern 1990). Another study verified that the microflora around clinically stable implants was similar to that of healthy gingival sulcus, and that microflora in peri-

implantitis were similar to that in periodontal pockets (Haanaes 1990).

Currently, titanium mini-plates as well as titanium mini-screws and dental implants have been applied as an absolute anchorage for tooth movement in orthodontic therapy, such as the skeletal anchorage system (SAS) (Umemori et al. 1999; Sugawara et al. 2002, 2004). However, approximately 10% of SAS anchor plate cases have developed acute inflammatory responses during orthodontic treatment (Nagasaka et al. 1999), and in the worst cases, the anchor plates were removed due to inflammation. Similarly, microbial flora in crevices around the anchor plates is one of the critical factors for the stability of implanted anchor plates and subsequent orthodontic treatment. However, there is no information on the nature of the microflora in anchor plate crevices under healthy and inflammatory conditions.

Therefore, the aims of this study were to characterize the microflora in crevices around titanium anchor plates using anaerobic culture for isolation of obligate anaerobes and molecular biological techniques for bacterial identification, as well as comparing the microbial composition between healthy and inflamed crevices.

Material and methods

Subjects

Seven periodontally healthy subjects with SAS using titanium mini-plates (age, 20–29 years; mean, 23.7 years) and three subjects with symptoms of swelling, pus discharge and spontaneous pain around the implanted titanium anchor plates at sampling (age, 23–25 years; mean, 24 years) were randomly selected for this study. In addition, seven periodontally healthy subjects (without SAS; age, 23–26 years; mean, 24.7 years) were also included in this study (Table 1).

Subjects were medically healthy by history, and received no antibiotics for 3 weeks preceding the sampling. Furthermore, they had neither anamnesis of pregnancy, genetic disease nor smoking habits.

Clinical oral examination

All subjects were examined for plaque accumulation by plaque index (Silness & Löe 1964), gingival inflammation by gingival index (Löe & Silness 1963) and probing depth using a periodontal pocket probe. Subjects were considered periodontally healthy based on clinical evaluation of plaque index ≤ 1 (Silness & Löe 1964) and gingival index ≤ 1 (Löe & Silness 1963), and no signs of acute inflammation; and there were no instances of probeable gingival sulcus depth greater than 3 mm or alveolar bone loss.

Sampling procedure

Sampling sites were isolated by cotton rolls, and supramucosal or supragingival plaque was carefully removed with a sterilized spoon excavator and pledget. Fluids from each transmucosal pocket (of seven healthy anchor plate crevices and three inflamed anchor plate crevices) around SAS implanted on zygomatic buttress, of which arms were placed beside the upper first molars (Fig. 1) were sampled with a micropipet, as described previously (Uematsu & Hoshino 1992). In addition, from seven periodontally healthy subjects, samples were taken from the bottom of the gingival crevice of proximal sites of the upper first molars.

Isolation of bacteria

Samples were transported in tightly screw-capped vials and were transferred as soon as possible (within a few minutes) to an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% N_2,

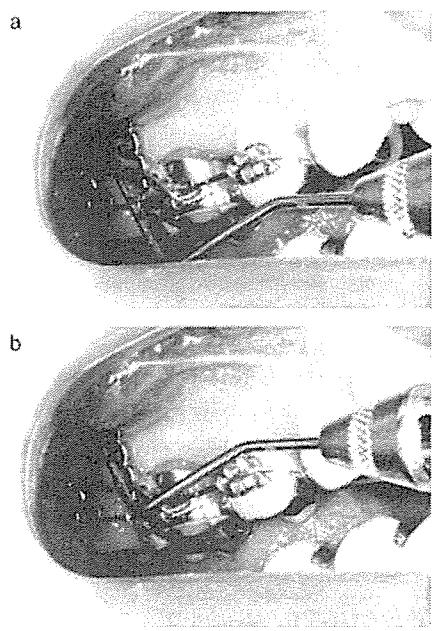

Fig. 1. Intraoral photograph of (a) an implanted titanium orthodontic anchor plate and (b) a periodontal pocket probe inserted into a transmucosal pocket.

Table 1. **Clinical features of subjects in this study**

	Healthy crevices with plates								Inflamed crevices with plates				Healthy gingival crevices (without plates)							
	1	2	3	4	5	6	7	Mean	8	9	10	Mean	11	12	13	14	15	16	17	Mean
Age	24	23	25	20	20	29	25	23.7	24	25	23	24	23	25	24	26	24	26	25	24.7
Gender	F	F	F	F	F	F	F	–	F	F	F	–	F	F	F	F	F	F	F	–
Loading periods*	1.1	1.2	0.9	1.0	1.6	0.9	0.7	1.1	0.8	2.9	3.4	2.4†	–	–	–	–	–	–	–	–
Sampling periods‡	2.9	4.2	3.7	3.6	4.3	5.9	4.6	4.2	9.5	4.7	28.4	14.2	–	–	–	–	–	–	–	–
Total implantation periods§	9.3	26.5	19.3	21.6	13.4	36.8	21.7	21.2	23.9	27.3	42.2	31.1	–	–	–	–	–	–	–	–
Sampling site	R	R	L	R	L	L	R	–	L	L	L	–	R	L	L	R	L	L	R	–
Probing depths (mm)	7	6	7	4	5	4	4	5.3¶	10	10	8	9.3¶,†	3	2	2	2	2	2	2	2.1
Plaque index	1	0	0	0	1	0	0	0.3	3	1	0	1.3¶	0	0	0	0	0	0	0	0
Gingival index	1	0	0	1	1	0	1	0.6	4	2	2	2.7¶,†	0	0	0	0	0	0	0	0

*Loading periods (months) by skeletal anchorage system.
†Significantly different ($P<0.05$) from the healthy crevices with plates.
‡Sampling periods (months) after implantation of titanium orthodontic anchor plates.
§Total implantation periods (months) after implantation of titanium orthodontic anchor plates.
¶Significantly different ($P<0.05$) from the healthy gingival crevices (without plates).
R and L indicate that samples were taken at the upper right and left molars sites, respectively.

10% H_2 and 10% CO_2. In the box each sample was suspended in 1 ml of sterilized 40 mM potassium phosphate buffer (pH 7) and dispersed with a teflon homogenizer. Serial 10-fold dilutions (0.1 ml each, from 10^{-4} to 10^{-6}) were spread onto the surface of Fastidious Anaerobe Agar (FAA, Lab M, Bury, UK) plates (duplicate) supplemented with 5% rabbit blood (Nippon Bio-Test Laboratories, Tokyo, Japan) and incubated in the anaerobic glove box at 37°C for 7 days. All plates, media, buffer solutions and experimental instruments were kept in the anaerobic glove box for at least 24 h before use. To ensure strictly anaerobic conditions in the glove box, reduction of methylviologen (−446 mV) was carefully checked whenever the experimental procedures were carried out. After incubation for 7 days, all colonies from plates having fewer than 100 colonies were subcultured.

Identification

Anaerobes and facultative anaerobes

Subcultured colonies were incubated anaerobically or aerobically for 3 days, and in this study, anaerobes were defined as bacteria which grew only in the anaerobic glove box, and facultative bacteria as those which also grew in air containing 30% CO_2, as described previously (Uematsu & Hoshino 1992; Sato et al. 1993).

DNA extraction and PCR-RFLP of 16S rRNA genes

Subcultured colonies were harvested by centrifugation at 7700 *g* for 5 min and the supernatants were removed. Genomic DNA was then extracted from the pellets with the InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions.

The 16S rRNA gene sequences were amplified by PCR using universal primers 27F and 1492R (Lane 1991) and Taq DNA polymerase (HotStarTaq Master Mix, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R, 5′-TAC GGY TAC CTT GTT ACG ACTT-3′. Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed as follows: 15 min at 95°C for initial heat activation and 35 cycles of 1 min at 94°C for denaturation, 1 min at 52°C for annealing, and 1.5 min at 72°C for extension and 10 min at 72°C for final extension. The 16S rRNA genes were individually digested with *Hpa*II or *Hae*III (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's instructions. Digestion products were separated on 2% agarose gels (High Strength Analytical Grade Agarose, Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4), stained with ethidium bromide and photographed under UV light. The molecular size marker was a 100 bp DNA Ladder (Invitrogen Corp., Carlsbad, CA, USA).

16S rRNA gene sequencing

Isolates were identified tentatively according to RFLP analysis (Sato et al. 1997, 1998a, 1998b, 2000, 2003; Sato & Kuramitsu 1999), and representative isolates were conclusively identified by sequence analysis as follows. The PCR products obtained above were sequenced at Hokkaido System Science Co. Ltd (Sapporo, Japan) using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100, Applied Biosystems Japan Ltd, Tokyo, Japan). Primers 27F and 1492R were used to sequence both strands (at least 1000 bp), and the partial 16S rRNA gene sequences were then compared with 16S rRNA gene sequences from the GenBank database using the Blast search program through the website of the National Center for Biotechnology Information. Bacterial species were determined by percent sequence similarity (>99%).

Data analysis

Fisher's exact probability tests and Tukey's tests were used to analyze significance. P values of <0.05 were considered statistically significant.

Results

Probing depths and gingival index of the inflamed anchor plate crevices were respectively greater than those of the healthy anchor plate crevices and healthy gingival crevices (Table 1). The plaque indices of the inflamed crevices were significantly greater than those of the healthy gingival crevices (Table 1). There were no significant differences in subject's age among the three crevices (Table 1). The average periods of loading by SAS were 1.1 and 2.4 months, sampling periods after implantation of titanium orthodontic anchor plates were 4.2 and 14.2 months, and total implantation periods after implantation of titanium orthodontic anchor plates were 21.2 and 31.1 months, in periodontally healthy subjects and inflammatory subjects, respectively. There were no significant differences in sampling and total implantation periods between the two groups, although the loading periods were longer in the inflammatory subjects than in the healthy subject (Table 1).

The average total colony-forming units (logarithm CFUs/ml) were 6.84 ± 0.85, 7.51 ± 0.76 and 8.88 ± 0.46 in healthy anchor plate crevices, inflamed anchor plate crevices, and healthy gingival crevices, respectively (Table 2), and significant differences were seen between healthy plate crevices and healthy gingival crevices, and between inflamed crevices and healthy gingival crevices. The amounts of bacteria were significantly lower in anchor plate crevices than in healthy gingival crevices (Table 2).

Table 2 shows the bacterial diversity in healthy and inflammatory anchor plate crevices and healthy gingival crevices. Of 184 strains isolated from healthy anchor plates crevices, 108 (59%) were anaerobic bacteria, while 73 (40%) were facultative bacteria. The predominant genera were *Actinomyces* (32 isolates, 17%), *Campylobacter* (22 isolates, 12%), *Fusobacterium* (19 isolates, 10%), *Selenomonas* (19 isolates, 10%) and *Streptococcus* (15 isolates, 8.2%). Of 133 strains isolated from inflamed anchor plate crevices, 110 (83%) were anaerobic and 17 (13%) were facultative bacteria. The predominant genera were *Prevotella* (62 isolates, 47%), *Fusobacterium* (22 isolates, 17%), *Campylobacter* (21 isolates, 16%) and *Eikenella* (10 isolates, 7.5%). On the other hand, of 146 strains isolated from healthy gingival crevices, 98 (67%) were facultative and 45 (31%) were anaerobic bacteria. The predominant genera were *Actinomyces* (54 isolates, 37%) and *Streptococcus* (29 isolates, 20%).

Discussion

The amounts of bacteria in anchor plate crevices were significantly lower than in

Table 2. **Bacterial isolates from crevices with orthodontic anchor plates and healthy gingival crevices**

	Healthy crevices with plates								Inflamed crevices with plates				Healthy gingival crevices (without plates)							
	1	2	3	4	5	6	7	Total	8	9	10	Total	11	12	13	14	15	16	17	Total
Log (CFU/ml)	7.84	6.34	5.81	6.81	7.85	5.94	7.31	(6.84)*,†	6.75	7.51	8.27	(7.51)*,†	9.23	9.41	8.15	8.45	8.74	9.20	8.98	(8.88)*
Total of isolation	16	29	25	14	29	18	53	184	92	16	25	133	22	24	16	22	19	20	23	146
Anaerobes	8	17	9	7	27	12	28	108 (59)‡	82	12	16	110 (83)†	20	1	7	2	6	2	7	45 (31)
Atopobium	1							1 (0.5)				0								0
Campylobacter	1		6	2	3	3	7	22 (12)	3	3	15	21 (16)			1		1		1	3 (2.1)
Dialister	1				4		1	6 (3.3)	2	1		3 (2.3)	1							1 (0.7)
Fusobacterium	3			1	14	1		19 (10)	17	4	1	22 (17)	1					1	2	4 (2.7)
Leptotrichia		8		3			3	14 (7.6)				0								0
Megasphaera								0				0						1		1 (0.7)
Micromonas								0				0	2		1				1	4 (2.7)
Mogibacterium	1							1 (0.5)				0	1							1 (0.7)
Olsenella								0				0	9							9 (6.2)
Peptostreptococcus								0				0	1							1 (0.7)
Prevotella	1	4	1		2	3	2	13 (7.1)	59	3		62 (47)	5				2			7 (4.8)
Selenomonas				1	4		14	19 (10)	1	1		2 (1.5)		1					2	3 (2.1)
Tannerella			2					2 (1.1)				0								0
Veillonella		5				5	1	11 (6)				0			5	2	3		1	11 (7.5)
Facultative anaerobes	7	12	15	7	1	6	25	73 (40)	7	4	6	17 (13)†	2	22	9	19	13	17	16	98 (67)
Actinomyces	4	2	4				22	32 (17)	2		2	4 (3)		20	7		12	1	14	54 (37)
Capnocytophaga		1				1		2 (1.1)				0		1	1				1	3 (2.1)
Eikenella				4				4 (2.2)	2	4	4	10 (7.5)								0
Gemella	1	1						2 (1.1)				0						3		3 (2.1)
Granulicatella		1						1 (0.5)				0						1		1 (0.7)
Haemophilus			4		1			5 (2.7)				0		1		1				2 (1.4)
Neisseria			7	3		2		12 (6.5)				0				1		2		3 (2.1)
Rothia								0				0					1	2		3 (2.1)
Streptococcus	2	7				3	3	15 (8.2)	3			3 (2.3)	2		1	17		8	1	29 (20)
Unidentified	1	0	1	0	1	0	0	3 (1.6)	3	0	3	6 (4.5)	0	1	0	1	0	1	0	3 (2.1)

*Mean log (CFU/ml) are given in parentheses.

†Significantly different ($P<0.05$) from the healthy gingival crevices (without plates).

‡Percentages are given in parentheses.

healthy gingival crevices (Table 2). In addition, amounts in inflamed anchor plate crevices were higher than in healthy anchor plate crevices, although the differences were not significant (Table 2). Anaerobic bacteria were predominant in anchor plate crevices, particularly in inflamed crevices, when compared with healthy gingival crevices. Crevices around dental implants with clinically healthy status have been reported to be similar to gingival crevices in the terms of bacterial density, proportion of anaerobes in bacterial flora, amount of fluid and profile of crevicular fluid constituents (Mombelli et al. 1987; Apse et al. 1989; Adonogianaki et al. 1995). In addition, upon challenge with bacteria on tooth and implant surfaces, inflammatory and immune responses of peri-implant mucosa have been reported to be similar to those of gingiva (Seymour et al. 1989; Tonetti et al. 1995; Liljenberg et al. 1997; Karoussis et al. 2004). In our study, however, bacterial density was lower in healthy plate crevices and the proportion of anaerobes among bacterial flora was higher than in healthy gingival crevices (Table 2) and crevices around dental implants (Mombelli et al. 1987). These results suggest that crevices around anchor plates differed from healthy gingival crevices and crevices around dental implant in the terms of amount and constituents of crevicular fluid, as well as inflammatory and immune responses.

The anatomical structure of crevices around anchor plates is different from that of gingival tissue and peri-implant tissue, and the anchor plates receive a continuous orthodontic force. Thus, anchor plate crevices may have sparse tissue structure and high secretion of tissue exudates. This situation may increase immune responses around the anchor plate and efflux of crevicular fluid, thus resulting in decreased bacterial density.

Our results showed that the loading periods were longer in the inflammatory subjects than in the healthy subject (Table 1), however, further studies on the relationship between loading periods and the microflora/inflammation are required.

In healthy gingival crevices, saccharolytic and facultative anaerobic bacteria, such as *Actinomyces* and *Streptococcus*, were predominant (Table 2), thus suggesting that the environment of healthy gingival crevices is fluctuant to pH and oxygen concentration. On the other hand, in healthy anchor plate crevices, anaerobic bacteria such as *Campylobacter*, *Fusobacterium* and *Selenomonas* were dominant in addition to *Actinomyces* and *Streptococcus* (Table 2). In inflamed anchor plate crevices, *Prevotella* and *Fusobacterium* were predominant (Table 2). These results suggest that anchor plate crevices are more anaerobic than healthy gingival crevices. Titanium anchor plates have deep crevices (4–7 mm) with clinically healthy status (Table 1) and crevices seemed to be largely shielded from atmospheric oxygen. Thus, anchor plate crevices may form an anaerobic environment and subsequently increase the proportion of anaerobic bacteria.

The presence of saccharolytic bacteria, i.e., *Actinomyces* and *Streptococcus* species, in healthy anchor plate crevices implies that the environment turns acidic upon sugar fermentation. However, *Fusobacterium* species, *Prevotella nigrescens* and *Prevotella intermedia*, detected in anchor plate crevices are known to be able to grow under acidic conditions and neutralize acidic environments by metabolizing amino acids and proteins (Takahashi & Schachtele 1990; Takahashi et al. 1997; Takahashi 2003). It has also been reported that the pathogenicity of *P. nigrescens* and *P. intermedia* increased in the absence of glucose via elevated proteolytic activity and production of cytotoxic end products (Saito et al. 2001). These results suggest that *P. nigrescens* and *P. intermedia* harbored in anchor plate crevices may increase their pathogenicity, particularly when the crevices are deep and the main nutrition source is nitrogenous compounds, such as proteins and amino acids.

In summary, this study suggests that the environment in crevices around titanium orthodontic anchor plates is anaerobic and supports anaerobic growth of bacteria, which may trigger inflammation in the tissue around the plates. Therefore, orthodontic treatment with titanium anchor plates requires strict self-care and regular professional plaque control in order to prevent infection.

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要旨

本研究では、細菌同定のための嫌気培養と分子生物学的手法を用いて、チタン製矯正用アンカープレート周囲溝内の細菌叢の構成を明らかにし、アンカープレート周囲溝と歯肉溝の細菌構成の比較を行った。被験者（20－29歳）のチタン・アンカープレート周囲溝（10名）と臨床的に健康な歯肉溝（7名）から採取した試料を嫌気条件で培養し、分離した細菌を16S rRNAシークエンス法により同定した。健康なアンカープレート周囲溝、炎症を伴うアンカープレート周囲溝および健康な歯肉溝内の、平均CFUs（log値）/mLは、それぞれ6.84、7.51、8.88であり、アンカープレート周囲溝の細菌密度は健康な歯肉溝より低いことが示された。7名の健康なアンカープレート周囲溝から分離された184菌株のうち、108（59%）は嫌気性菌であり、73（40%）は通性菌であった。このうち、*Campylobacter*（12%）、*Fusobacterium*（10%）、*Selenomonas*（10%）などのグラム陰性桿菌、*Actinomyces*（17%）、*Streptococcus*（8.2%）などのグラム陽性通性菌が優勢であった。3名の炎症を伴うアンカープレート周囲溝から分離した133菌株のうち、110（83%）は嫌気性菌であり、*Prevotella*（47%）、*Fusobacterium*（33%）、*Campylobacter*（16%）などのグラム陰性桿菌が優勢であった。一方、7名の健康な歯肉溝から分離した146菌株のうち、98（67%）は通性菌であり、45（31%）は嫌気性菌であった。これらの結果から、チタン製矯正用アンカープレート周囲溝内の環境は嫌気的であり、嫌気性菌の増殖が促進されることによって、プレート周囲の歯周組織の炎症が惹起される可能性が示唆された。

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