J Med Dent Sci 2006; 53: 159−166

Original Article

Role of oral streptococci in the pH-dependent carious dentin

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The aim of the present study was to investigate the extent to which oral streptococci or lactic acid producing bacteria are able to regulate pH value, especially at low pH values associated with dentinal carious activity using a PCR method. One millimeter-thick sections were sliced from 24 extracted carious human molars. The pH values on the tooth sections were evaluated using a pH-imaging microscope. A dentin sample (1×1×1mm) was prepared from the areas with the lowest or highest pH and homogenized to extract bacterial genomic DNA. Specific primers were used for nested PCR to mutans streptococci (MS: *Streptococcus mutans* and *Streptococcus sobrinus*). Conserved primers were also used for PCR to lactobacilli and gram positive bacteria. The PCR products were separated by electrophoresis, and then oral bacteria were identified. There was no significant difference between carious and intact dentin in MS identification. However, the frequency of the product amplified by the conserved primers in carious dentin (16/24, 66.7%) was significantly higher than that in intact dentin (2/24, 8.3%), and PCR products demonstrated, by sequence analysis, various bacteria, including oral streptococci. It was concluded that oral streptococci may be associated with the development of "low pH-carious dentin".

Key words: pH, carious dentin, *S. mutans*, PCR, oral streptococci

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Received May 2; Accepted June 9, 2006

Introduction

Mutans streptococci (MS) and lactobacilli have been associated with the initiation and progression of dental caries[1-4]. Individuals with active caries lesions harbor significantly greater numbers of MS when recently established colonies are analysed on enamel surfaces, whereas "caries-inactive" individuals showed significantly higher proportions of *Streptococcus sanguis* colonies and IgA1 protease produced by streptococci[5]. Van Houte and colleagues[6-8] have addressed the role of acidogenic bacteria, other than MS and lactobacilli, in the root surface caries process and have demonstrated that non-MS and *Actinomyces* spp. are heterogeneous with respect to their acidogenicity. Their observations suggested that the relative proportions of the different species in subjects varied widely for oral flora on enamel to root surfaces for risk of caries. It is important to precisely define the distribution of bacteria in dentin caries lesions by the selection and increase of acidogenic organisms. This is equally important for enamel and root surface caries. The progression of dental caries into the tooth is

different from that of caries at the tooth surface with respect to the contribution of cariogenic bacteria. Organic acids produced by micro-organisms play an important role, not only in demineralizing the inorganic components of a tooth, but also in enhancing bacterial penetration[9] and proteolytic destruction of the organic components of a tooth[10].

Acidogenesis at low pH seems to be an important cariogenic bacterial trait[7]. Acidogenicity and aciduranse, the ability to generate acid and to function at low pH, appear to be the main physiological traits associated with the cariogenic nature of these organisms[11]. None of the studies referred to have shown whether the bacterial population shifts were caused by a higher affinity of certain organisms for carbohydrates or by the greater sensitivity of certain species to a low pH.

Recently, a pH-value analyzing technique has been introduced to cariology studies that uses a pH-imaging microscope (SCHEM-100, Horiba Ltd, Kyoto, Japan). The pH values of carious and intact dentin have been investigated visually and quantitatively[12,13]. The pH distribution of carious dentin was shown to be lower than that of intact dentin using this pH-imaging microscope. A comparison was made between pH value distribution and lesions stained with a caries detector solution[12]. Another comparison of pH and mineral-loss was performed on active and arrested caries lesions[13], which showed a significant correlation between a pH decrease and mineral loss in active carious dentin lesions.

However, it is unclear whether pH is a conclusive indicator for active or inactive dentin caries. The important role of changes in pH in localized dentin regions is accompanied with bacteria producing lactic acid, and is believed to play a role in caries activity. Therefore, the progress of cariology research using pH imaging analysis and polymerase chain reaction (PCR) methods to detect oral microorganisms is likely to provide a better understanding of the processes in caries dentin. The aim of the present study was to investigate the extent to which oral streptococci or lactic acid producing bacteria are able to regulate pH value, especially at low pH values associated with dentinal carious activity using a PCR method.

Materials and Methods

Sample teeth

Twenty four extracted human molars with moderate-to-severe dentin caries on the occlusal surface were used for this study. The teeth were obtained from different individuals requiring extractions as part of their treatment (8 males, 16 females; age 26.6±4.7 yr, 29.9 ±7.8 yr, overall mean; age 28.8±7.1 yr) and consented verbally to using their teeth for research. The extracted teeth were stored frozen at −20°C in acid-base characteristics for no longer than one month to avoid alterations until the experimental procedure. After the roots were removed, the center of each caries lesion in the occlusal region was vertically sliced using a diamond saw (Leitz Instruments, Heidelberg, Germany) to produce a section approximately 1-mm thick (Fig. 1A). Each sliced surface was ground with 600-grit silicon carbide paper under running water for 10 strokes.

Evaluation of the pH distribution using pH-imaging microscope

For distinction between carious and intact dentin, the pH values of the sectioned surfaces were measured with the pH-imaging microscope (SCHEM-100) which has a flat silicon sensor and displays the pH distribution of specimens as a pH image. For evaluating pH distribution, the specimens require intact flat surfaces. The pH distribution formed on a thin agar film made on the sensor can be measured quantitatively, and a pH dependent electric signal at each measurement point is converted and displayed as a pH image (Fig. 1B). When a sample contacts the agar film, H^+ or OH^- ions travel from the dentin surfaces into the agar film. The amounts of these ions are calculated into grayscale pixels which are then arranged to form a pH image by image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). On the pH image, the low intensity grayness shows low pH values, and high intensity grayness shows high pH values.

In this study, the caries lesion or intact dentin was classified using pH-imaging analysis. The area of low intensity grayness was determined to be the carious dentin lesion, and the area of high intensity grayness was to be the intact dentin[12].

Preparing sample fragments from pH measured teeth

By analyzing the image, the area exhibiting the lowest pH and the highest pH area was identified in each tooth. From one section of a tooth, two sample fragments (1×1×1mm) were obtained using the sterilized diamond bur; one was a carious dentin sample and the other was an intact dentin sample (Fig. 1C). To maintain the clear operation filed under the sampling, new ster-

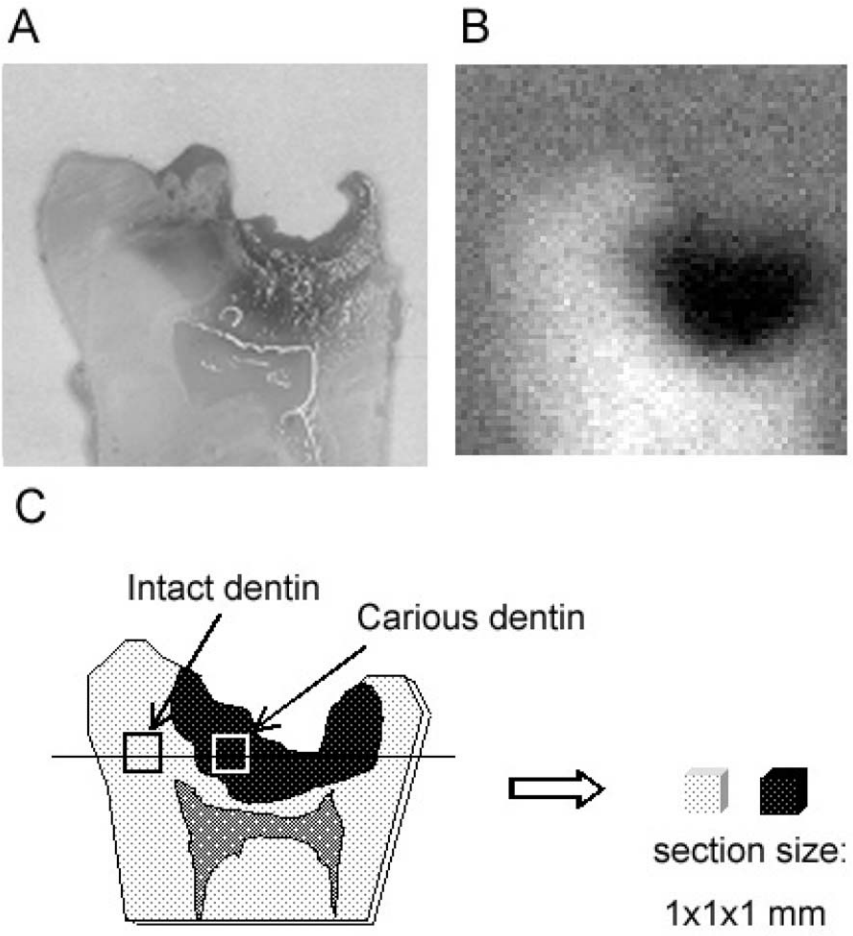

Fig. 1. Sample preparation
A: One mm thick sectioned sample was prepared from a tooth with carious dentin.
B: The pH-image of SCHEM-100 is shown in the sliced samples. The lowest pH area was identified in the low intensity area (black) which shows carious dentin and the highest pH area in high intensity area (white) which shows intact dentin.
C: Sections (1×1×1mm) were prepared from the lowest pH zone in carious dentin and highest pH zone in intact dentin.

ilized burs and air-turbine heads were used for each tooth in this study. A total of 24 pairs from the carious and intact dentin were obtained from each extracted tooth. Each sample was kept in microcentrifuge tubes and stored frozen at −20°C until the DNA extraction procedures were performed.

Bacterial strains for PCR analysis

The bacterial strains used as the positive or negative control for PCR analysis were *S. mutans* MT8148, *S. sobrinus* 6715, and *Lactobacillus casei* ATCC393. MS are facultative anaerobes and were cultured on brain heart infusion (BHI, Difco Laboratories, Detroit, MI, USA) agar plates anaerobically for 2d. One of the colonies was selected and picked up from the culture plates and grown at 37°C in BHI broth overnight. *L. casei* was cultured on BHI-blood agar plates under anaerobic conditions for 1 d and after this, colonies were selected from the culture plate, they were grown at 37°C in Lactobacilli deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) overnight.

Extraction of chromosonal DNA

Chromosomal DNA from bacteria was extracted as follows. Bacterial cells grown in BHI or MRS broth were centrifuged and suspended in sterile PBS solution twice. After centrifugation, the supernatant was care-

fully exposed. The bacterial pellet was dissolved with an enzyme lysis soloution and stored at 37°C for over 30 min. DNA of each bacteria was extracted using a DNA extraction kit (DNeasy Tissue Kit, QIAGEN, Tokyo, Japan) according to the manufacturer's instructions.

Isolation of bacterial DNA from sample teeth sections

Before the DNA extraction, sample teeth fragments were homogenized using a homogenizer (Mikro-Dismembrator U, B. BRAUN Biotech international, PA, USA). After homogenization of each fragment, the enzyme lysis solution was added and stored 37°C for over 30 min. Genomic DNA was extracted using a DNA extraction kit as above.

Polymerase chain reaction (PCR) experiments

In order to confirm *S. mutans* and *S. sobrinus* molecular identities, DNA from isolated MS was submitted to a two-step strategy PCR method, using primers specific for portions of the glucosyltransferase genes described by Oho *et al* [14]. The sequences of the primers are listed in Table1. For the first PCR, primers GTFB-F and GTFB-R were used to amplify the *gtfb* sequence of *S. mutans,* and GTFI-F and GTFI-R were used to amplify the *gtfI* sequence of *S. sobrinus*. The conserved primers LARNA5 and LARNA6, which were selected on the basis of the comparison of the available 16s rRNA sequences of lactobacilli and gram positive bacteria, were also used to detect lactobacilli or gram positive bacteria for PCR[15]. Each PCR mixture (25μl) comprised 2.5 μl of either dentin sample DNA or bacterial genomic DNA, 2.5 μl of the primers (0.5μM each), 12.5 μl of Premix Taq (TAKARA BIO INC., Shiga, Japan), and 5 μl of DW. PCR amplification was performed in a PCR thermal cycler (PTC-200, MJ Research, Watertown, MA, USA) using the following cycling program: DNA polymerase activation at 95.5°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. To improve the detection level of MS, nested PCR was performed using internal primers[14]. The primers were GTFB-FIN and GTFB-RIN to *S. mutans*, and GTFI-FIN and GTFI-RI to *S. sobrinus* (Table 1). The products (1μl) obtained from the first PCR amplification were used as the template for the nested PCR. Nested PCR was performed under the same conditions as the first PCR, except for the template solution volume. Positive and negative controls were included in each PCR set and for all sample processing. The PCR products were separated by electrophoresis on 2% agarose gel, and then total bacteria were identified by electrophoresis size and DNA sequence.

Sequence analysis

The sequence of PCR products of the sample DNA strains were analyzed by Shimadzu Biotech (Shimadzu Co., Kyoto, Japan) and checked in sequence using the DDBJ Homology Search System (http://spiral.genes.nig.ac.jp/homology/top-e.html). Furthermore, the sequence of the products from the laboratory strains used as the positive controls (*S. mutans, S. sobrinus* and *L. casei*) was confirmed.

Statistical analysis

All the collected data were statistically analyzed by Peason's Chi-square test and Fisher's extend test. Data were analyzed using the Statistical Package for Medical Science (SPSS Ver.11 for Windows) for statistical procedures.

Table 1. PCR primers

Primers	Sequence	Location
GTFB-F	5'-ACTACACTTTCGGGTGGCTTGG	793 - 814
GTFB-R	5'-CAGTATAAGCGCCAGTTTCATC	1288 - 1309
GTFI-F	5'-GATAACTACCTGACAGCTGACT	871 - 892
GTFI-R	5'-AAGCTGCCTTAAGGTAATCACT	1561 - 1582
GTFB-FIN	5'-AAAGCAGATTCTAATGAATCGA	817 - 838
GTFB-RIN	5'-AATGTAAAATTTTGCCATCAGC	1264 - 1285
GTFI-FIN	5'-TGGTATCGTCCAAAATCAATCC	895 - 916
GTFI-RIN	5'-AGATTTGCAGTTGGTCAGCATC	1537 - 1558
LARNA5	5'-GTTGTCCGGATTTATTGGG	574 - 592
LARNA6	5'-GGGTATCTAATCCTGTTCGC	821 - 802

Results

pH measurement of sample teeth

The pH values for each sample are listed in Table 2. The pH value of carious dentin (range pH 5.9 to 6.4) was less than that of intact dentin (range pH 6.6 to 7.2). The lowest pH point was always detected at the inner region from the periphery of pH-imaging carious characterization[12]. Fig. 1 shows a photograph of a representative case of a caries lesion (Fig. 1A) and its SCHEM-100 image (Fig. 1B). Two dentin fragments (1×1×1 mm) were extracted from the lowest-pH and highest-pH areas of each tooth (Fig. 1C).

PCR analysis and bacterial detection

S. mutans and *S. sobrinus* in the carious and intact dentin samples were detected using the nested PCR method[14]. Lactobacillus or gram positive bacteria in carious and intact dentin samples were also detected by the PCR method using the conserved primers. Fig. 2 shows the representative results of nested PCR to MS (Fig. 2A) and PCR to Lactobacillus or gram positive

Table 2. The results of pH values and bacteria detection

	Carious dentin				Intact dentin			
No.	pH value	*S. mutans*	*S. sobrinus*	Total bacteria	pH value	*S. mutans*	*S. sobrinus*	Total bacteria
1	5.9			+	6.7			
2	5.9			+	6.7	+		
3	6.0				6.7	+		
4	6.0	+		+	6.6	+		
5	6.0	+	+	+	6.7			
6	6.1	+		+	6.7	+		
7	6.1	+		+	6.8	+		
8	6.2			+	6.8			
9	6.2				6.8			
10	6.2	+		+	6.7	+		
11	6.2				6.8			
12	6.3	+	+	+	7.0			
13	6.3			+	6.7		+	+
14	6.3	+	+	+	6.6			
15	6.3		+	+	6.9	+	+	
16	6.3				6.9			
17	6.3				6.8		+	
18	6.3				6.7			
19	6.4	+	+	+	7.0	+		+
20	6.4			+	6.8			
21	6.4				6.8			
22	6.5			+	7.4			
23	6.5				6.9			
24	6.6	+		+	7.2			
total		9	5	16		8	3	2

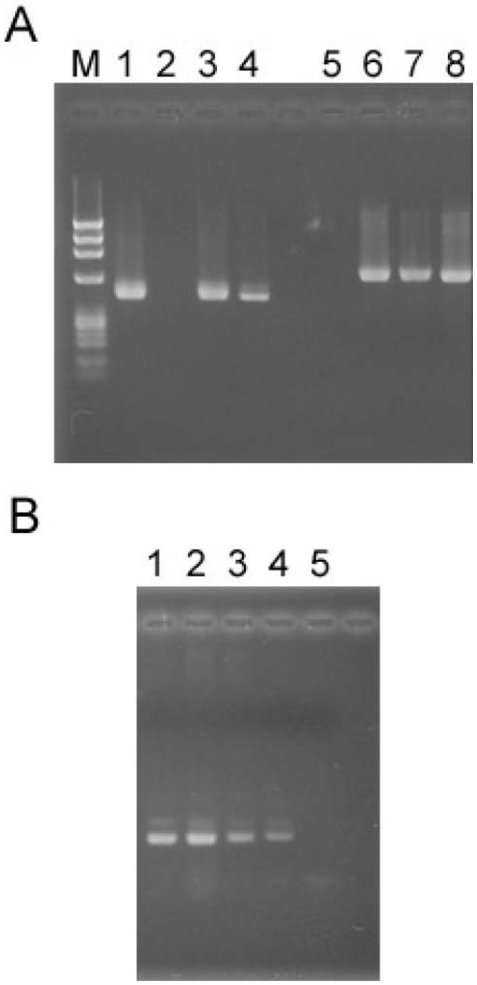

Fig. 2. PCR analyses in samples from the dentin fragments
A: Typical appearance of detection of nested PCR products using *S. mutans* and *S. sobrinus* inner primers was performed in the electrophoresis. DNA from *S. mutans* MT8148; positive control (1 and 5 lanes), *S. sobrinus* 6715; positive control (2 and 6), lowest pH sample section (3 and 7) and highest pH sample section (4 and 8) were used for PCR analyses (*S. mutans* and *S. sobrinus*); M: *Hae* —*III* digested DNA ladder.
B: Photograph showing detection of PCR products using the conserved primers was performed by electrophoresis. DNA from *S. mutans* MT8148; positive control (lane 1), *S. sobrinus* 6715; positive control (2), *L. casei* ATCC393; positive control (3), lowest pH sample section (4), and highest pH sample section (5).

bacteria (Fig. 2B). The products using the conserved primers were recognized in the positive controls, *S. mutans*, *S. sobrinus*, and *L. casei*. The results of bacterial detection are shown in Table 2. From carious dentin, *S. mutans* was detected in 9 samples, and *S. sobrinus* in 5 samples, and Lactobacillus or gram positive bacteria in 16 samples. On the other hand, in the intact dentin, *S. mutans* was detected in 8 samples, *S. sobrinus* in 3 samples, and Lactobacillus or gram positive bacteria in 2 samples. The results of sequence analysis of PCR products amplified by conserved primers indicated the presence of various streptococci and other bacteria, such as Enterococcus, Listeria, Lactococcus, and Bacillus strains (data not shown). The results demonstrated the identification of whole bacteria. Statistical analysis showed that there was not a significant difference between carious and intact dentin for *S. mutans* or *S. sobrinus* identification ($p > 0.05$). However, the frequency of the all PCR product amplified by the conserved primer (16/24, 66.7%) in carious dentin was significantly higher than that (2/24, 8.3%) in intact dentin ($p < 0.0001$).

To observe if a relationship existed between lowest pH in carious dentin and penetration of *S. mutans* into intact dentin, the caries was divided into 2 groups (sample with the pH ≥ 6.3 and pH ≤ 6.2). *S. mutans* was detected in 6 of 11 intact dentin samples (54.5 %) with the pH ≤ 6.2 in caries dentin. In contrast, *S. mutans* was detected in 2 of 13 intact dentins (15.3%), where the pH ≥ 6.3 in carious dentin. Although there was no significant difference between ≤ 6.2 and ≥ 6.3 ($p = 0.082$), detection rate in intact dentin with the pH ≤ 6.2 (54.5 %) was higher than the pH ≥ 6.3 (15.3%).

Discussion

Recently, the pH values of extracted carious and intact dentin have been investigated visually and quantitatively using a pH-imaging microscope[12]. The pH distribution in carious dentin was shown to be lower than that of intact dentin. In addition, there was a significant correlation between pH decrease and mineral loss in active dentinal caries lesions[13]. The pH imaging microscope seems to be useful for determining active or arrested caries as the carious dentin seems to show pH-dependent variation. This quantitative measurement for caries diagnosis may also aid dentists to assess changes in caries activity over time. In addition, sample preparation for the pH-imaging microscope does not require destruction or dissolution of a specimen. Therefore, a dentinal caries lesion examined with the pH-imaging microscope can show the lowest and highest pH values throughout, and the same sample could be used to determine if a relationship exists between microbiological penetration/presence and pH.

Many studies have shown that MS can be isolated in greater numbers from caries lesions[3,4]. Generally, MS

play a central role in the development of biofilm and dental caries[3,11]. MS produce insoluble glucans, which help incorporate other bacteria, and enhance the formation of mature biofilm via the quorum sensing system[16,17]. The quorum sensing system is a cell-to-cell signaling mechanism used to regulate cellular processes in a cell density-dependent manner of various bacterial strains. Recent work by the same group indicated that the sensing system is related to acid tolerance[18,19]. Current knowledge indicates that the optimal development of acid tolerance in *S. mutans* requires both a low pH induction and cell-to-cell communication. Biofilm cells of *S. mutans* in a biofilm-chemostat were shown to be significantly more acid tolerant than planktonic cells in the same chemostat and they (*S. mutans*) induced an acid tolerance response, albeit at a slower rate than that of exponential-phase batch-grown cells[20]. In the present study, it was not possible to detect clear differences in *S. mutans* and *S. sobrinus* composition for the highest pH and lowest pH samples of intact and carious dentin. However, all the lowest pH carious dentin samples detected *S. mutans* accompanied with total bacteria, but 7 out of 8 the highest pH intact dentin samples detected only *S. mutans*. On the other hand, the percentage of the detection rate (16 out of 24 cases, 66.7%) of total bacteria in lowest pH lesion of carious dentin was significantly higher than that (2 out of 24 cases, 8.3%) in the highest pH sections of intact dentin. *S. mutans* seems to induce the dentin destruction which is closely associated with the formation of glucan-mediated large aggregates of *S. mutans* and other bacteria[21]. Therefore, *S. mutans* may form a biofilm in the dentin, and incorporate and collaborate with various bacteria for induction of acidulating the dentin, enable acid tolerance and create what is regarded as active caries.

Recent studies have recovered other streptococcal species from plaque including, e.g., *S. mitis*, *S. gordonii*, *S. anginosus* and *S. oralis*[22-24]. Even in the case of advanced dentin caries, the highest proportion of total bacteria, including oral streptococci, was detected in the lowest pH dentin sample in this study. Because these species have been reported to be prevalent among the colonizers of teeth[5], they could play an important role in preparing the environment to make it suitable for the outgrowth of MS. Local conditions of a low pH may create favorable conditions for the proliferation or penetration of biofilm bacteria such as *S. mutans* and *S. sobrinus*. In the present study, *S. mutans* alone was detected at a higher rate (55%) in the intact dentin in the same tooth, when the lowest pH was less than 6.2 in the adjacent carious dentin. Although there was no significant difference, when the lowest pH was greater than 6.3 in carious dentin, fewer *S. mutans* were observed (15%). Oral streptococci can infect and penetrate into dentin tubules when a sample is etched with a strong acid, such a phosphoric acid, and smear layer completely removed[9,25]. It is hypothesized that the acidogenic activity of the bacteria may provide the opportunity for penetration of *S. mutans* from carious dentin into intact dentin especially for an active caries lesion.

Taken together, pH imaging analysis and the PCR analysis to detect oral microorganisms in dentin caries were important for determining active caries and also the demonstration of penetration and infection by cariogenic bacteria into intact dentin causing lesion progression. This information could be applied for the therapeutic treatment of caries lesions during minimally invasive cavity preparation[26,27] rather than surgically excising caries.

Acknowledgements

This project was supported by Grant #14207079, #17390507 and #18592083 from the Japan Society for the Promotion of Science, and for Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone in Tokyo Medical and Dental University. The authors would like to thank Drs Mizuho Motegi and Masaomi Ikeda for their helpful advice in preparing the manuscript.

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