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Sonic extracts from a bacterium related to periapical disease activate gelatinase A and inactivate tissue inhibitor of metalloproteinases TIMP-1 and TIMP-2

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Abstract

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Aim To examine the effects of sonicated bacterial extracts (SBEs) from three related to periapical disease bacteria (*Porphyromonas gingivalis*, *P. endodontalis* and *F. nucleatum*) on the activation of matrix metalloproteinase (MMP-2) and the inactivation of tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-2).

Methodology Each SBE was added to cultures of human periodontal ligament (PL) cells or HT1080 cells and their supernatants were analysed by zymography for MMP-2. Each SBE was added to PL cell cultures, and the amount of TIMP-1 was determined by ELISA. *P. gingivalis* SBE was incubated with HT1080 cell culture supernatants, and the amounts of TIMP-1 and TIMP-2 were determined by ELISA. Statistical analysis was performed with the paired Student's *t*-test.

Results In extracts of PL cells that had been incubated in the presence of *P. gingivalis* SBE, one representing pro-MMP-2 (72 kDa) and a band corresponding to the active MMP-2 (66 kDa) were observed; but in the other extracts it was not detected. When HT1080 cells were treated with *P. gingivalis* SBE, the pro-MMPs was processed into 86- and 66-kDa fragments, but in the other extracts, the processing did not occur when the other SBEs were used. When PL cells were incubated with the same SBEs, the amount of TIMP-1 was markedly decreased ($P < 0.01$), but in the other extracts, it was not. The amounts of both TIMP-1 and TIMP-2 were decreased in a dose-dependent manner when HT1080 cell culture supernatant was incubated with *P. gingivalis* SBE.

Conclusions These findings suggest that *P. gingivalis* SBE may cause connective tissue to be destroyed, contributing to the process of periapical disease, by activating pro-MMP-2 as well as by inactivating TIMP-1 and TIMP-2.

Keywords: ECM, MMP-2, *P. gingivalis*, SBE, TIMP-1, TIMP-2.

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Introduction

Inflammatory changes in major pathological lesions in oral tissues could progress via the destruction of the extracellular matrix (ECM) in the periodontal ligament and alveolar bone. It has been reported that the degradation of the ECM is associated, in large part, with matrix metalloproteinases (MMPs), including interstitial collagenase (MMP-1), gelatinase A (72-kDa gelatinase/IV type collagenase, MMP-2), gelatinase B (92-kDa gelatinase/type IV collagenase, MMP-9) and stromelysin-1 (MMP-3). Twenty-three members of this MMP family have been detected in humans (Visse & Nagase 2003). These MMPs are also produced and

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secreted by inflammatory cells, including neutrophils and macrophages that migrate into inflamed sites, as well as by noninflammatory cells, including fibroblasts (Nagase & Woessner 1999). Four tissue inhibitors of metalloproteinases (TIMPs), the common endogenous inhibitors of these MMPs have been reported: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Declerk *et al.* 1989, Goldberg *et al.* 1989, Stetler-Stevenson *et al.* 1989, Kishi *et al.* 1991, Ward *et al.* 1991, Apte *et al.* 1994, Uria *et al.* 1994, Green *et al.* 1996). These TIMPs play roles in controlling the degradation of ECM components by inhibiting MMP activity; the imbalance between levels of MMPs and TIMPs during inflammation is thought to be of importance (Nagase & Woessner 1999). Study of infected root canal systems using modern anaerobic bacterial culture techniques has revealed many obligate anaerobic bacteria (Bergenholtz 1974, Kantz & Henry 1974, Wittgow & Sabiston 1975, Brook *et al.* 1981, Oguntebi *et al.* 1982, Williams *et al.* 1983, Haapasalo 1989) including *Porphyromonas*, *Bacteroides*, *Prevotella*, *Fusobacterium*, *Eubacterium*, and *Veillonella* species (Sundqvist 1992). ECM-degrading enzymes, including collagenase, hyaluronidase, and LPS (endotoxic activity) produced by such bacteria have been reportedly involved in the development and progress of periapical diseases (Higerd *et al.* 1978, Singer & Dutton 1979, Nair *et al.* 1983, Berit & Klaus 1986).

In this study, sonicated bacterial extracts (SBEs) from three obligate anaerobic bacteria (*P. endodontalis*, *Porphyromonas gingivalis*, and *F. nucleatum*) were used to examine their effects on gelatinase A (MMP-2), TIMP-1, and TIMP-2, which are known to be present in periapical tissue, by using human periodontal ligament (PL) cell cultures and human fibrosarcoma (HT1080) cell culture supernatants.

Materials and methods

Preparation of human periodontal ligament cell cultures

Three freshly extracted human teeth for orthodontic reasons were immediately soaked and washed with sterilized physiological saline containing penicillin (1000 U mL^{-1}; Meiji Seika Kaisha, Ltd, Tokyo, Japan) and Fungizone (30 μg mL^{-1}; Nippon Squib, Tokyo, Japan). Periodontal ligament tissue was then isolated from the surfaces of the roots and washed thoroughly with RPMI1640 (Nisui Pharmaceuticals, Tokyo, Japan) containing penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}; Meiji Seika Kaisha) and Fungizone (3 μg mL^{-1}). It was then minced into small pieces of approximately 1.5 mm × 1.5 mm; and the pieces were placed in a 35-mm cell culture dishes (Corning, NY, USA), containing RPMI1640 medium supplemented with 10% foetal bovine serum (FBS; Immuno-Biological Laboratories Co. Ltd, Saitama, Japan), penicillin (100 U mL^{-1}), kanamycin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}), prior to being cultured at 37 °C in a 5% CO_2 atmosphere. When the cells migrating from the pieces of tissue became confluent, they were subcultured and used for experiments at subculture levels 4–8.

Bacteria used and preparation of sonicated bacterial extracts

Three obligate anaerobic gram-negative bacteria, *P. endodontalis* ATCC 35406, *P. gingivalis* 381, and *F. nucleatum* ATCC 10953, were cultured in an anaerobic glove box (Sanyo, Tokyo, Japan) at 37 °C and harvested at the late log phase of growth. The cells were harvested by centrifugation at 5000 *g* for 15 min, washed twice with PBS, suspended in PBS again, and then sonicated twice for 9 min each time in an icebox by using a Sonifer 250 (Taikex Co., Saitama, Japan). Thereafter, the sonicates were centrifuged at 10 000*g* for 30 min, the intact cells were removed, and the supernatants were collected. After the supernatants had been sterilized by passing through a 0.45-μm filter (Corning), the protein content of each SBE (unfractionated) was determined with a protein assay kit (Bio-Rad, Tokyo, Japan; data is not shown). Then all supernatants were diluted to a concentration of 1 mg of protein per millilitre and used for further experiments as sonicated bacterial extracts.

Preparation of human fibrosarcoma (HT1080) cell culture supernatants

HT1080 cells were cultured with RPMI1640 medium containing 10% Fetal Bovine Serum (FBS) in 100-mm cell culture dishes at 37 °C in a 5% CO_2 atmosphere. When they had become confluent, they were washed twice with PBS, which was then replaced with serum-free RPMI1640 medium, and cultured for a further 48 h. Then, their supernatants were collected and centrifuged at 3000 *g* for 5 min to remove cell components, before being centrifuged further at 10 000 *g* for 20 min. The supernatants produced were used as samples for experiments.

Preparation of TIMP-1 and TIMP-2

TIMP-1 was purified from human gingival cell culture medium according to the previous method (Kodama *et al.* 1981); and TIMP-2, as reported earlier (Sakamoto *et al.* 1996).

Cell culture and SBE response experiment

The present study was conducted to examine the MMP-2-activating ability of and the amounts of TIMP-1 and TIMP-2 produced by PL cells and HT1080 cells in the presence of *P. gingivalis*, *F. nucleatum*, and *P. endodontalis* SBEs. PL cells (adjusted to 1.5×10^5 cells mL^{-1} in RPMI1640 medium containing 10% FBS) were divided into 24-well culture plates (Funakoshi, Tokyo, Japan), each well containing 1 mL, and allowed to grow to confluence at 37 °C in a 5% CO_2 atmosphere. They were then washed twice with PBS followed by replacement with 1 mL of serum-free RPMI1640 medium containing 10 μg mL^{-1} of SBE. The cells were subsequently cultured for 48 h and supernatants were collected and centrifuged at 3000 *g* for 5 min to remove cell components, before being centrifuged further at 10 000 *g* for 20 min. The supernatants produced were used as samples for experiments and were used to determine MMP-2 activity and the amounts of TIMP-1 and TIMP-2.

Treatment of cell culture supernatants with SBEs

One millilitre of HT1080 cell culture supernatant was added to 1 mL of each SBE, and the mixture was incubated at 37 °C for 18 h.

Determination of MMP-2 activity

Gelatin zymography was used to determine MMP-2 activity. Electrophoresis was conducted according to the previous method (Laemmli 1970). The acrylamide concentrations of the gel were 3% (condensing gel) and 10% (separating gel). The separating gel had gelatin added to it (DIFCO, Lakes, NJ, USA) to a final concentration of 0.3 mg mL^{-1}. Tris–glycine buffer containing 0.1% sodium dodecyl sulphate (SDS) was used as the running buffer. After electrophoresis, the gel was soaked in a 2.5% TritonX-100 solution, and shaken twice at room temperature for 30 min to remove the SDS. The gel was then soaked in a 30 mmol L^{-1} Tris sodium chloride buffer containing 5 mmol L^{-1} $CaCl_2$ and 0.2 mol L^{-1} NaCl (pH 7.5; refer buffer A below) before being shaken twice at room temperature for 30 min. Then the TritonX-100 was removed, and the gel was incubated at 37 °C for 24 h. Finally, the gel was stained with Coomassie brilliant blue R (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) at room temperature for 1 h before being decolorized with 5% methanol–7.5% acetic acid. This procedure allowed the MMP-2 activity to be observed as clear bands on a blue background. For the experiments, Pre-stained Standards Low Range for SDS-PAGE (Bio-Rad) was used as molecular weight markers, and HT1080 cell culture supernatants were used as the positive control.

Determination of amounts of TIMP-1 and TIMP-2

The amounts of TIMP-1and TIMP-2 were determined by using a previously reported Enzyme Immunoassays (EIA) method (Kodama *et al.* 1990, Fujimoto *et al.* 1993).

Determination of TIMP-1 inhibition of MMP-1 and MMP-2

The TIMP-1 inhibition of MMP-1 and MMP-2 was by the solution method (Terato *et al.* 1976) with [^{14}C]glycine-labelled collagen used as substrate.

The TIMP-1 inhibition of MMP-2 was also determined by reverse zymography. After electrophoresis and gel treatment in the same way as for gelatin zymography, active gelatinase was added to buffer A (1 U mL^{-1}); and the resultant gel was incubated at 37 °C for 24 h. Then the gel was stained with Coomassie brilliant blue R at room temperature for 1 h and decolorized with 5% methanol–7.5% acetic acid. In this way, TIMP-1 was observed as a blue band.

Treatment of MMP-2 or TIMP-1 with SBEs

Purified MMP-2 (0.5 μg mL^{-1}) or purified TIMP-1 (0.4 μg mL^{-1}) was incubated with *P. gingivalis* SBE (0.1 μg mL^{-1}) at 37 °C for 18 h.

Statistical analysis

All measurements were made in triplicate, and the average values were calculated for each group. Data were expressed as mean ± standard deviation of the means (SD; $n = 3$ for each group). Differences between control and experimental treatment groups were determined by using the paired Student's *t*-test. Differences were considered significant if $P < 0.01$.

Results

Processing by sonicated bacterial extracts of progelatinase A into a 66-kDa fragment

Progelatinase A in periodontal ligament cell culture medium
Periodontal ligament cell culture medium was treated with each SBE and subjected to gelatin zymography. As shown in Fig. 1, neither *F. nucleatum* nor *P. endodontalis* SBE at 10 μg mL^{-1} showed any effect on the 72-kDa single band corresponding to progelatinase A (pro-MMP-2), the density of which was no different from that of the control. In the presence of 10 μg mL^{-1} *P. gingivalis* SBE; however, the band corresponding to pro-MMP-2 completely disappeared. By reducing the concentration of *P. gingivalis* SBE down to 1 μg mL^{-1}, two bands with molecular masses of 72- and 66-kDa were detected.

Progelatinase A in HT1080 cell culture medium
Electrophoresis of HT1080 cell culture medium produced 92- and 72-kDa bands, which corresponded to gelatinase B (pro-MMP-9) and pro-MMP-2, respectively (Fig. 2, lane 2). Only *P. gingivalis* SBE processed both pro-MMPs, pro-MMP-9 into an 86-kDa fragment and pro-MMP-2 into a 66-kDa fragment (lane 3). Neither *F. nucleatum* nor *P. endodontalis* SBE had any effect on either pro-MMP (lanes 5 and 7). None of these SBEs alone possessed MMP-processing activities for either MMP (lanes 4, 6, and 8).

Purified progelatinase A

Purified pro-MMP-2 was processed by *P. gingivalis* SBE. As shown in Fig. 3, two bands, corresponding to 72-kDa pro-MMP-2 and its 66-kDa fragment, were detected by gelatin zymography.

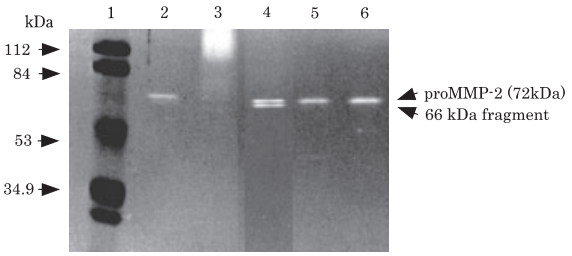

Figure 1 Gelatin zymograph of periodontal ligament (PL) cell culture medium treated with sonicated bacterial extracts (SBEs) at 37 °C for 48 h. 1, molecular weight markers; 2, PL cell culture medium alone; 3, 2 + *Porphyromonas gingivalis* SBE (10 μg mL^{-1}); 4, 2 + *P. gingivalis* SBE (1 μg mL^{-1}); 5, 2 + *F. nucleatum* SBE (10 μg mL^{-1}); 6, 2 + *P. endodontalis* SBE (10 μg mL^{-1}).

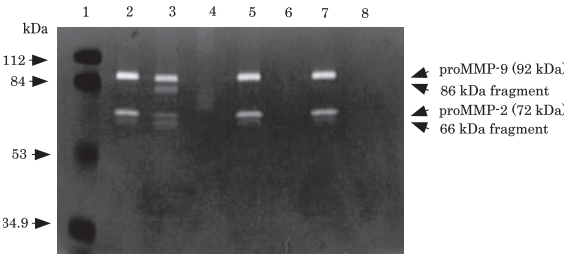

Figure 2 Gelatin zymograph of HT1080 cell culture medium treated with SBEs at 37 °C for 18 h. 1, molecular weight markers; 2, HT1080 cell culture medium alone; 3, 2 + *Porphyromonas gingivalis* SBE(1 μg mL^{-1}); 4, *P. gingivalis* SBE(1 μg mL^{-1}) alone; 5, 2 + *F. nucleatum* SBE(10 μg mL^{-1}); 6, *F. nucleatum* SBE(10 μg mL^{-1}) alone; 7, 2 + *P. endodontalis* SBE (10 μg mL^{-1}); 8, *P. endodontalis* SBE (10 μg mL^{-1}) alone.

Amount of TIMP-1 in PL cell culture medium

Periodontal ligament cell culture medium was first incubated with a given SBE, and the medium was then subjected to a sandwich EIA for TIMP-1. TIMP-1 was not detected in the culture medium of cells treated with *P. gingivalis* SBE, suggesting that TIMP-1 was degraded by *P. gingivalis* SBE (Fig. 4). Neither the SBE from *F. nucleatum* nor that from *P. endodontalis* affected the amount of TIMP-1 in the culture medium.

Dose-dependent degradation of TIMP-1 and TIMP-2

P. gingivalis SBE at different concentrations degraded both TIMPs dose-dependently, as shown in Fig. 5.

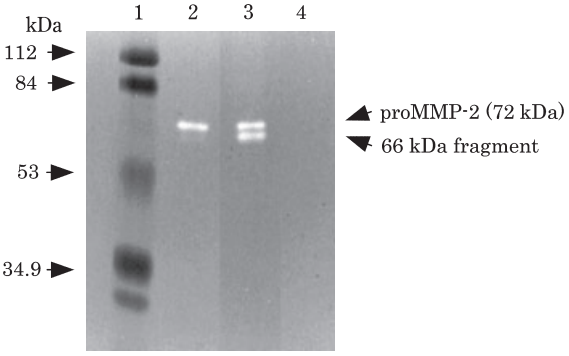

Figure 3 Gelatin zymograph of purified progelatinase A (pro-MMP-2) treated with *Porphyromonas gingivalis* SBE at 37 °C for 48 h. 1, molecular weight markers; 2, pro-MMP-2 alone; 3, 2 + *P. gingivalis* SBE (1 μg mL^{-1}); 4, *P. gingivalis* SBE (1 μg mL^{-1}) alone.

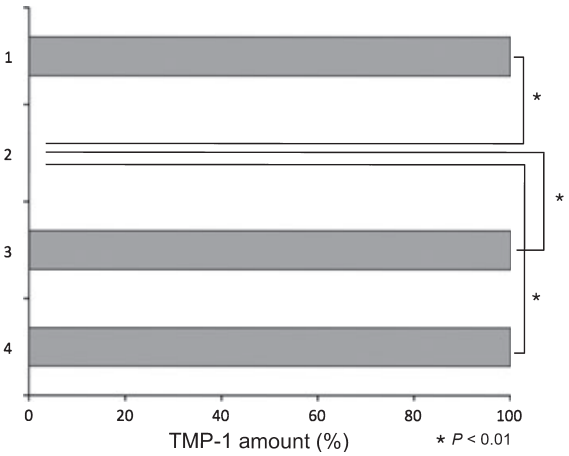

Figure 4 Effects of SBEs on the amount of TIMP-1 in PL cell culture medium. PL cell culture medium was first treated with 10 μg mL^{-1} of each SBE at 37 °C for 48 h, and then was subjected to the sandwich EIA for TIMP-1. 1, PL cell culture medium alone; 2, 1 + *Porphyromonas gingivalis* SBE; 3, 1 + *F. nucleatum* SBE; 4, 1 + *P. endodontalis* SBE.

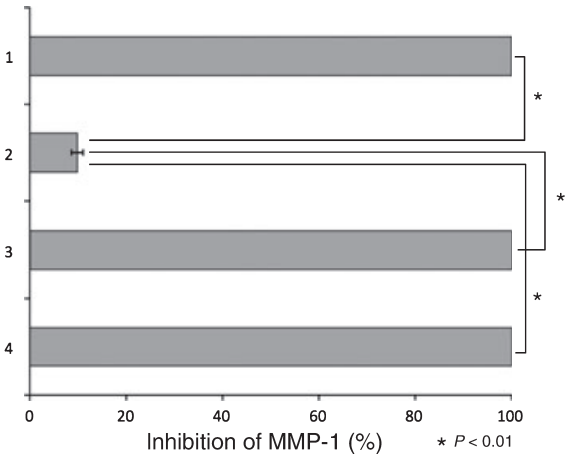

Figure 6 Effects of SBEs on the inhibitory activity of purified TIMP-1 against interstitial collagenase (MMP-1). Purified TIMP-1 (0.4 μg) was first with 0.1 μg of each SBE at 37 °C for 48 h, and then was subjected to the inhibition assay for MMP-1. *Porphyromonas gingivalis* SBE alone significantly reduced the inhibitory activity of TIMP-1 toward MMP-1 to about the 100% of the control ($P < 0.01$). 1, TIMP-1 alone; 2, 1 + *P. gingivalis* SBE; 3, 1 + *F. nucleatum* SBE; 4, 1 + *P. endodontalis* SBE. *$P < 0.01$.

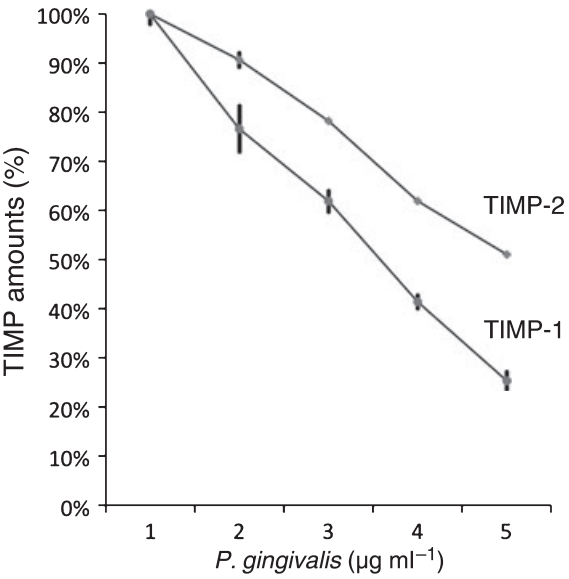

Figure 5 Dose-dependent degradation by *Porphyromonas gingivalis* SBE of TIMP-1 and TIMP-2 in HT1080 cell culture medium. HT1080 cell culture medium was first treated with 1 μg mL^{-1} of *P. gingivalis* SBE at 37 °C for 48 h, and then EIAs for TIMP-1 and TIMP-2 were performed. Amounts of TIMPs are expressed as a percentage of the amount in the culture medium without treatment.

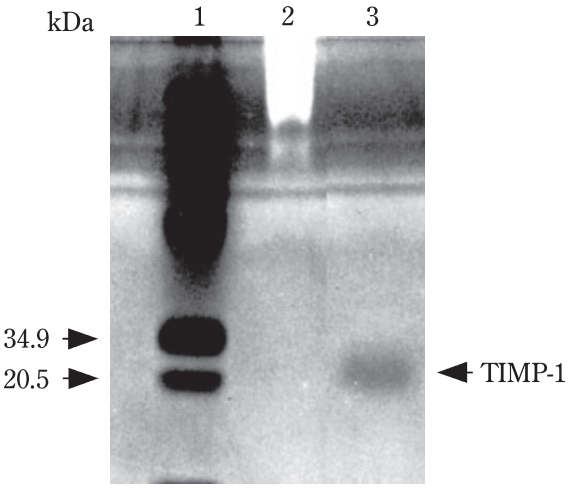

Figure 7 Reverse gelatin zymograph of purified TIMP-1 treated with *Porphyromonas gingivalis* SBE. The same sample prepared for the inhibition assay for MMP-1 shown in Fig. 6 was subjected to reverse zymography. 1, molecular weight markers; 2, 3 + *P. gingivalis* SBE (0.1 μg mL^{-1}); 3, TIMP-1 (0.4 μg mL^{-1}) alone.

TIMP-1 was more susceptible to *P. gingivalis* SBE than TIMP-2.

Inhibitory activity of TIMP-1

The effect of each SBE on the inhibitory activity of TIMP-1 against interstitial collagenase (MMP-1) was

examined. *P. gingivalis* SBE alone significantly reduced the inhibitory activity of TIMP-1 toward MMP-1 to about the 100% of the control (Fig. 6; $P < 0.01$). The effect of *P. gingivalis* SBE on the inhibitory activity of purified TIMP-1 was further confirmed by reverse zymography of TIMP-1. As shown in Fig. 7, essentially no inhibition against MMP-2 was observed with purified TIMP-1 pre-treated with *P. gingivalis* SBE.

Discussion

One of the types of enzyme that could be of importance in the degradation of ECM is the MMP family. The collapse of the balance between MMPs and their common endogenous inhibitors, TIMPs, is an important point in understanding the progress of connective tissue destruction. It is known, however, that most MMPs are secreted from cells as inactive pro-enzymes; therefore, MMPs are subject to two different processes: activation by an activator(s) and inactivation via TIMPs.

For activation of pro-MMP *in vivo*, proteases could be major factors, and trypsin, plasmin, cathepsin G, elastase, and MMP-3 have been shown to act as activators of MMPs. However, pro-MMP-2 is not activated by any of these proteases (Collier *et al.* 1988), and for a long time it was not known which activators could activate pro-MMP-2 *in vivo*. Recently, membrane-type MMP (MT-MMP) has been found to be activating substance that is specific for pro-MMP-2, and four subclasses of it have been reported. It has been elucidated that MT-MMP has a transmembrane domain, which other MMPs do not have, at its C-terminus and that MT-MMP specifically activates pro-MMP-2 on cell surfaces (Sato *et al.* 1994). The only pro-MMP-2 activator that has been reported is MT1-MMP, one of the MT-MMPs.

At the outset, it was believed that MMP-2 might be activated by bacteria related to periapical diseases. Studying pro-MMP-2 activation by exogenous factors including bacterial components could be useful for increasing understanding of the mechanisms of tissue destruction during inflammation, and could be also of help to clarify the development, progress, and mechanism underpinning the progression of periapical diseases.

In the current study, the MMP-2-activating ability of PL cells and HT1080 cells in the presence of *P. gingivalis*, *F. nucleatum*, and *P. endodontalis* SBEs was examined for the first time, although similar studies have been conducted with human periodontal ligament or human gingival fibroblasts in the presence of *P. gingivalis* supernatant (Pattamapun *et al.* 2003, Tiranathanagul *et al.* 2004, Zhou & Windsor 2006). Also, it is unclear whether TIMP-1 and TIMP-2 can be inactivated by *P. gingivalis*, although similar studies have been conducted (Grenier & Mayrand 2001, Tiranathanagul *et al.* 2004). In this study, MMP-2 in PL cell culture medium treated with *P. gingivalis* SBE was analyzed by gelatin zymography, and a band of pro-MMP-2 and a band that could be its active form were observed. In the samples with *P. gingivalis* SBE alone, no similar bands of gelatin-degrading activity were observed. The new band, which disappeared when Ca^{2+} was removed by Ethylen Diamine Tetra Acetic Acid (EDTA) (data not shown), could be a band derived from MMPs; and this suggests that it could be an active band produced from pro-MMP-2 being converted to be a smaller molecule by *P. gingivalis* SBE. The reason for the emergence of this band (molecular weight of 66 kDa) may be that *P. gingivalis* SBE first activates pro-MT1-MMP, and then the active MT1-MMP activates the pro-MMP-2 produced by PL cells. Therefore, similar experiments using a system without cells were planned. However, PL cell in primary culture only produce low amounts of pro-MMP-2 and TIMP-1 and have slow growth. To overcome these problems, HT1080 cell culture supernatants were used, because these cells have been studied in detail with regard to their production of pro-MMP-2, TIMP-1, and TIMP-2, and also because they are derived from fibroblasts, which are the same as PL cells. HT1080 cell culture supernatants were directly incubated with each SBE, and a band corresponding to a smaller molecule (66 kDa), which could be the active form of MMP-2, emerged along with a band representing pro-MMP-2 (72 kDa). In addition to that, treatment of purified pro-MMP-2 with *P. gingivalis* SBE resulted in a similar outcome. These results strongly suggest that pro-MMP-2 activation would not be associated with MT1-MMP, but with factors derived from *P. gingivalis* SBE. No 66-kDa bands were observed in the experiments using either *F. nucleatum* SBE or *P. endodontalis* SBE, therefore, the activating factors could be considered to be specific to *P. gingivalis*. When *P. gingivalis* SBE (10 μg mL^{-1}) was added to the PL cell culture system, TIMP-1 was not detected in the medium of PL cultures, whereas in the media of cultures treated with either *P. endodontalis* SBE or *F. nucleatum* SBE, the same amount of TIMP-1 as in the control was detected. These results suggest the following four possibilities: (i) *P. gingivalis* SBE could inhibit the production of TIMP-1 by PL cells, (ii) *P. gingivalis* SBE could inhibit and

suppress the secretion of TIMP-1 by PL cells, (iii) *P. gingivalis* SBE could degrade the TIMP-1 secreted from the PL cells, (iv) *P. gingivalis* SBE could kill PL cells. Amongst these, four was rejected because there was no cytotoxity against PL cells at the concentration of SBE used (data not shown). Regarding 1 and 2, when a cell culture medium was incubated with *P. gingivalis* SBE, the amount of TIMP-1 in the medium decreased in a dose-dependent manner; therefore, it is unlikely that the TIMP-1 production and secretion capabilities of the cells were impaired by *P. gingivalis* SBE. Therefore, the third possibility was suggested. To clarify this, purified TIMP-1 was reacted with *P. gingivalis* SBE, and the TIMP-1 inhibition of MMPs (for MMP-1 and MMP-2) was found to be decreased, which suggested that TIMP-1 could be degraded by *P. gingivalis* SBE (Grenier & Mayrand 2001).

For TIMP-2, the same experiments were conducted. When each SBE was added to PL cell cultures, the amount of TIMP-2 produced by PL cells was extremely small; therefore, the effects of *P. gingivalis* SBE could not be confirmed. However, in the experiments using HT1080 cell culture medium, the amount of TIMP-2 protein in the medium decreased in a *P. gingivalis* SBE dose-dependent manner, the same as the findings for TIMP-1, which suggests that TIMP-2 could also be degraded by *P. gingivalis* SBE.

It is well known that *P. gingivalis* possesses fimbriae and LPS and that it produces hemagglutinin and various proteases. It has recently been shown that a trypsin-like protease, the main type of these proteases, is one of the cysteine proteases termed gingipain and that there are two strains of gingipain (Arg-gingipain, Lys-gingipain), each having a different substrate specificity (Pike *et al.* 1994). It was also shown that gingipain has collagenolytic activity (Yamamoto 1995), and can activate pro-MMP-1 and pro-MMP-9 (DeCarlo *et al.* 1997). It was also confirmed that pro-MMP-9 was activated by *P. gingivalis* SBE (Fig. 2). Both pro-MMP-2 activation and TIMP-1, TIMP-2 inactivation were only confirmed by using the media treated with *P. gingivalis* SBE; however, it can not be concluded that these results were due to the same components of *P. gingivalis*. It is also unclear whether the results of experiments such as these conducted in cell cultures or *in vitro* will be the same *in vivo*. However, because obligate anaerobic bacteria are isolated in great numbers from infected root canals with progressing periapical lesions (Sundqvist 1992), the results obtained from these present experiments should be helpful for clarifying the development and progression of periapical disease. It would be worthwhile to determine the mechanisms of enzyme synthesis and activation of pro-MMP-2 and others, as well as the identity of the enzymes by Matrix Assisted Laser Desorbtion Ionization-Time of Flight-MS (MALDI-TOF MS) peptide fingerprinting in the future.

Conclusions

P. gingivalis SBE may contribute to the destruction of connective tissue in a developing periapical lesion by activating pro-MMP-2 as well as by inactivating TIMP-1 and TIMP-2, which may change the balance between MMPs and TIMPs in the ECM.

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