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Oral Biology

MMP-2 activation by *Actinobacillus actinomycetemcomitans* supernatant in human PDL cells was corresponded with reduction of TIMP-2

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OBJECTIVE: Matrix metalloproteinase 2 (MMP-2) has been implicated to play a role in pathogenesis of periodontal disease. We recently reported that *Porphyromonas gingivalis* supernatant could activate MMP-2 in human periodontal ligament (HPDL) cells. In this study, activation of MMP-2 by *Actinobacillus actinomycetemcomitans* supernatant and the mechanism was investigated.

METHODS: HPDL cells were treated with either *A. actinomycetemcomitans* or *P. gingivalis* supernatant for 48 h. To verify the mechanism, pretreated inhibitors were used. Gelatin zymography, RT-PCR and Western blot analysis were used to detect the activation of MMP-2, expression of MT1-MMP and TIMP-2 mRNA and the proteins, respectively.

RESULTS: The supernatant from *A. actinomycetemcomitans* could activate MMP-2 in HPDL cells similar to that from *P. gingivalis* but by a different mechanism. Activation by *A. actinomycetemcomitans* supernatant was correlated with a reduction of TIMP-2 secretion without any alteration of MT1-MMP, while activation by *P. gingivalis* increased MT1-MMP but no change of TIMP-2 was found.

CONCLUSION: The supernatant from *A. actinomycetemcomitans* and *P. gingivalis* could induce the activation of MMP-2 possibly through the imbalance of MT1-MMP and TIMP-2 in HPDL cells but by different mechanisms. The imbalance of MT1-MMP and TIMP-2 may be another factor that is involved in the severity of periodontal disease.
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Keywords: MMP-2; MT1-MMP; PDL cells; TIMP-2

Introduction

Periodontitis is a chronic inflammatory disease that leads eventually to loss of tooth supporting structures and to loss of teeth. It is well documented that the destruction of periodontal tissue is initiated by a group of gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Bacteroides forsythus* (Haffajee and Socransky, 1994; Zambon, 1996). Although these bacteria can directly destroy the periodontal tissue by their proteolytic enzymes, it is now widely accepted that the host response to these bacteria and their products, which increase the secretion of inflammatory cytokines and host proteolytic enzymes, is the major cause of the periodontal destruction (Birkedal-Hansen, 1993; DeCarlo *et al*, 1997).

Matrix metalloproteinases (MMPs) form a family of proteolytic enzymes that significantly participates in the destructive events of periodontal disease (Wolley and Davies, 1981). High levels of particular members of MMP, such as MMP-1, -3, -8 and MMP-9 had been found in the crevicular fluid of periodontitis patients (Sorsa *et al*, 1988; Birkedal-Hansen, 1993; Ingman *et al*, 1996; Romanelli *et al*, 1999; Soell *et al*, 2002). In addition, MMP-2 (72 kDa type IV collagenase) is another enzyme that was implicated to play a role in pathogenesis of periodontal disease. An increased level of MMP-2, especially the active form of the enzyme, was detected in the periodontal tissues of patients with periodontitis (Ingman *et al*, 1994, 1996; Mäkelä *et al*, 1994; Korostoff *et al*, 2000).

All members of the MMP family, including MMP-2, are secreted in a latent form and require activation in order to function. The process of activation is one of the important steps in the regulation of the MMP activity (Ellerbroek and Stack, 1999). Usually, activation of MMP can be achieved by a proteolytic cleavage of the MMP pro-peptide using a number of serine proteinases such as plasmin or furin (Nagase, 1997; Sternlicht and Werb, 2001). However, these serine proteinases cannot activate MMP-2. The activation process of MMP-2 occurs at the cell surface as proposed by Strongin *et al* (1995). In this model, pro-MMP-2 binds to membrane type 1-matrix metalloproteinase (MT1-MMP) by the facilitation of tissue inhibitor of matrix metalloproteinase 2

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(TIMP-2). Then, another molecule of MT1-MMP cleaves the pro-domain of pro-MMP2 into an intermediate form of MMP-2, which is immediately followed by an autocatalytic cleavage generating the fully active MMP-2. Hence the balance between MT1-MMP and TIMP-2 is an important requirement for the MMP-2 activation. The imbalance between the pro-form and active form of MMP-2 had been reported to correlate with some diseases such as cancer (Di Nezza *et al*, 2002; Waas *et al*, 2002) and possibly in the periodontal disease.

Our previous report showed that *P. gingivalis* supernatant could activate pro-MMP-2 in human periodontal ligament (HPDL) cells (Pattamapun *et al*, 2003). In addition, the activation was correlated with the up-regulation of MT1-MMP at both the transcription and translation levels following experimental treatment with the supernatant. In this study, we further investigated the effect of another periodontopathogenic bacterium, *A. actinomycetemcomitans*, in the activation of MMP-2.

Materials and methods

Cell culture

HPDL cells were cultured from the explants obtained from the periodontal ligament (PDL) attached to non-carious, freshly extracted third molars, or teeth removed for orthodontic reason as previously described (Pattamapun *et al*, 2003). All patients gave informed consent. The PDL tissues were scraped out from the middle third of the root and harvested on a 60-mm culture dish (Nunc, Napervile, IL, USA). The explants were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, and 5 $\mu g\ ml^{-1}$ amphotericin B at 37°C in humidified atmosphere of 95% air, 5% CO_2. Medium and the supplement were from Gibco BRL (Carlsbad, CA, USA). Cells from the third to the fifth passages were used.

All experiments were performed triplicate using cells prepared from three different patients.

Human osteosarcoma cell line, U2OS, and human dermal fibroblast cell line, HDF, were kindly provided by Professor Erik Thompson, St Vincent's Institute of Medical Research. Cells were cultured in DMEM supplemented with 10% fetal calf serum as described above.

Cultivation of bacteria

Actinobacillus actinomycetemcomitans (ATCC 43718) was cultivated in brain heart infusion broth (BHI; Difco, Sparks, MD, USA) at 37°C in 5% CO_2.

Porphyromonas gingivalis W50 (ATCC 53978) was grown at 37°C in an anaerobic jar (Oxoid® Laboratory, Becton Dickinson, Sparks, MD, USA) under anaerobic condition using Gas Pak® (BBL Microbiology System, Becton Dickinson) in tryptic soy broth (TSB, BBL Microbiology System, Becton Dickinson) supplemented with 1.5% yeast extract (Difco, Sparks, MD, USA), 5 $\mu g\ ml^{-1}$ hemin (Sigma Chemical Co., St Louis, MO, USA) and 0.2 $\mu g\ ml^{-1}$ menadione (Boehringer Mannheim GmbH, Indianapolis, IN, USA).

Both strains were grown until an optical density reached 0.6–0.7 at 660 nm. The bacterial cultures were centrifuged (10 000 *g*, 15 min, 4°C) and the supernatant was collected and filtered through a 0.2 μm filter membrane, then stored at −80°C until use.

Activation of HPDL fibroblasts with the supernatant

HPDL cells were seeded in 24-well plates (Nunc) at a density of 50 000 cells ml^{-1} per well and were allowed to attach for 16 h. After silencing the cells with serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co.) overnight, the supernatant from bacteria were added and incubated for another 48 h. All treatments were conducted in a serum-free condition and an equal amount of bacterial broth was added to the control. After 48 h, the medium was collected and kept at −20°C prior to the MMP-2 analysis.

In the inhibitory experiment, cells were treated with protease inhibitors for 30 min before the bacterial supernatant was added. The inhibitors used in the experiment included 10 μM phenanthroline, 5 ng ml^{-1} (0.25 TIU) aprotinin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 50 $\mu g\ ml^{-1}$ leupeptin. All inhibitors were obtained from Sigma Chemical Co.

Gelatin zymography

MMP-2 activity was evaluated by gelatin zymography. The medium was subjected to a 10% SDS-polyacrylamide gel containing 0.1% gelatin under a non-reducing condition as described previously (Pattamapun *et al*, 2003). The latent and active MMP-2 can be detected as clear bands at positions 72 and 62 kDa, respectively.

Reverse-transcription polymerase chain reaction

Cells were seeded in six-well plates at a density of 25 000 cell cm^{-2} and treated with *P. gingivalis* or *A. actinomycetemcomitans* supernatant as described above. After 48 h, total cellular RNA was extracted with Trizol (Gibco BRL) according to the manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by a reverse transcription using an Avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) for 1.5 h at 42°C. Subsequent to the reverse transcription, a polymerase chain reaction was performed. The primers specific to MT1-MMP, TIMP-2 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were prepared by GENSET (Genset Biotech, Singapore) following the reported sequences from GenBank (TIMP-2;GI:9257247, MT1-MMP;GI:4826833, GAPDH;GI:4503912). The oligonucleotide sequences of MMP-2, MT1-MMP, TIMP-2 and GAPDH primers were:

MT1 MMP: sense 5′ CATCGCTGCCATGCAGA AGT 3′
antisense 5′ GTCATCATCGGGCAGCAC 3′
TIMP-2: sense 5′ GGAAGTGGACTCTGGAAAC GACATT 3′
antisense 5′ CTCGATGTCGAGAAACTCCTGC TTG 3′

GAPDH: sense 5′ TGAAGGTCGGAGTCAACG GAT 3′
antisense 5′ TCACACCCATGACGAACATGG 3′

The PCR was performed using Tac polymerase with a PCR volume of 25 μl. The reaction mixtures contained 25 pmol of primers and 1 μl of RT product. The PCR working conditions were set at a denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C, and chain elongation for 1.45 min at 72°C on a DNA thermal cycler (ThermoHybaid, Ashford, UK). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining.

Western blot analysis of MT1-MMP and TIMP-2
Cells were seeded in 6-well plates (Nunc) and treated with supernatant of the bacteria. The condition medium was collected and centrifuged to remove cell debris for TIMP-2 analysis. MT1-MMP and tubulin were analyzed from the cell extract using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing a cocktail of protease inhibitors (Sigma Chemical Co.). The amount of protein was determined by BCA^{TM} protein assay (Pierce, Rockford, IL, USA). All samples, 25 μg of total protein per lane, were subjected to electrophoresis under a reducing condition on a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The antibodies for TIMP-2 and MT1-MMP (the affinity-isolated antibody against the TIMP-2 C-terminal and the affinity-isolated antibody against the MT1-MMP hinge region) were obtained from Sigma Chemical Co. The anti-tubulin antibody was a gift from Professor Erik Thompson, St Vincent's Institute of Medical Research. All antibodies were diluted in 5% non-fat milk. After staining with the primary antibody, the membrane was subsequently incubated with the biotinylated secondary antibody for 30 min, followed by another 30 min staining with peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA). After extensive washes with PBS, the membrane was coated for the chemiluminescence detection (Pierce) for 1 min and the signal was captured with CL-Xposture film (Pierce). The amount of protein was determined using a densitometer (Imaging densitometer GS-700; BioRad, Hercules, CA, USA) and Molecular Analyst Software (BioRad).

Statistical analysis
All data were analyzed using a one-way analysis of variance (ANOVA). Scheffe's test was used for *post-hoc* analysis ($P < 0.05$).

Results

Supernatant of A. actinomycetemcomitans *activated MMP-2 in fibroblasts*
The dose of *A. actinomycetemcomitans* supernatant responsible for the MMP-2 activation in HPDL cells was determined. Figure 1a showed that the supernatant could activate MMP-2 (62 kDa) in a dose-dependent manner with an optimal dose of about 50 μl ml^{-1}. This result suggests that the response of HPDL cells in terms of MMP-2 activation depends on the concentration of secreted molecule(s) from *A. actinomycetemcomitans*.

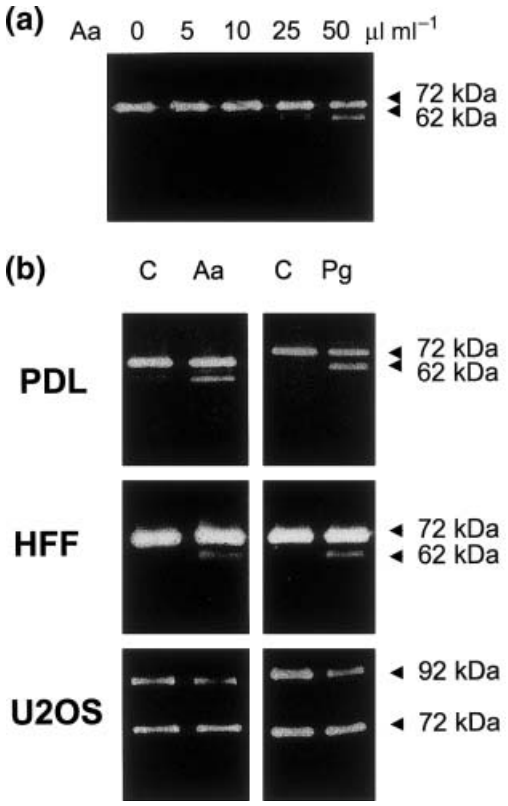

Figure 1 (**a**) HPDL cells were treated with 0, 5, 10, 25 and 50 μl ml^{-1} of supernatant for 48 h and the activation of MMP-2 was analyzed by gelatin zymography. The activation could be detected clearly when treated with 50 μl ml^{-1}. (**b**) For the gelatin zymography analysis, HPDL cells, human dermal fibroblast (HDF) and human osteosarcoma cell line (U2OS) were treated with either 10 μl ml^{-1} of *P. gingivalis* supernatant or 50 μl ml^{-1} of *A. actinomycetemcomitans* supernatant for 48 h. An equal amount of bacterial broth was added to the control. MMP-2 activation could be detected in HPDL and HDF, but not in U2OS, when cells were treated with both types of the supernatant. Positions of the latent and active MMP-2 (72 and 62 kDa) are indicated on the right

To examine if the effect on the activation depends on certain cell types, we tested the ability in MMP-2 activation on human dermal fibroblasts (HDF) and human osteosarcoma cell line (U2OS). Cells were grown and treated with 50 μl ml^{-1} of *A. actinomycetemcomitans* or 10 μl ml^{-1} of *P. gingivalis* supernatant for 48 h. The results showed that supernatant of both *A. actinomycetemcomitans* and *P. gingivalis* could induce MMP-2 activation in HPDL cells as well as in HDF but not in U2OS (Figure 1b). The lack of ability to activate MMP-2 secreted by U2OS suggesting the specific target of secreted molecule(s) from *A. actinomycetemcomitans*.

In addition, we also found that the supernatant from *Escherichia coli*, *Streptococcus salivarius* and *Fusobacterium nucleatum* did not contain the ability of MMP-2 activation in HPDL cells (data not shown) that also indicates the specificity of the supernatant from *A. actinomycetemcomitans* in MMP-2 activation.

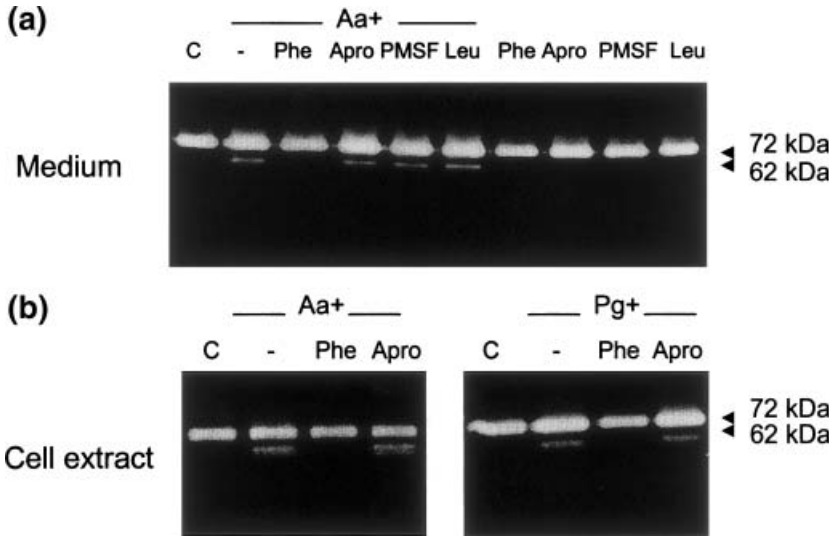

Figure 2 (**a**) Gelatin zymography showed the inhibitory effect of protease inhibitors. HPDL cells were treated with 50 μl ml^{-1} of *A. actinomycetemcomitans* (Aa) supernatant in the presence or absence of protease inhibitors; phenanthroline (Phe), aprotinin (Apro), PMSF and leupeptin (Leu). The figure shows that the activation of MMP-2 could be inhibited only by phenanthroline. Inhibitors alone did not have any effects on the activation. Fifty microliters per ml of BHI was added to the control (c). (**b**) The MMP-2 activation could be detected in cell extracts after treatment with *A. actinomycetemcomitans* (Aa) or *P. gingivalis* (Pg) supernatant. Similarly, the activation was also inhibited by phenanthroline. Positions of the latent and active MMP-2 (72 and 62 kDa) are indicated on the right

Different kinds of protease inhibitors were used to investigate the possible pathway for MMP-2 activation by *A. actinomycetemcomitans*. Phenanthroline, an MMP inhibitor, aprotinin and PMSF, serine protease inhibitors, and leupeptin, a cysteine protease inhibitor were each introduced to the culture media 30 min prior to the *A. actinomycetemcomitans* supernatant and followed by another 48-h incubation. The result revealed that the activation of MMP-2 by *A. actinomycetemcomitans* in the culture medium could be inhibited only by phenanthroline as shown in Figure 2a. The results indicate that the activation of MMP-2 by *A. actinomycetemcomitans* supernatant is MMP-dependent. In addition, an increased level of active MMP-2 was also detected on the cell surface suggesting an association of activated MMP-2 on the surface of HPDL cells, which imply the functional MMP-2. Application of phenanthroline also inhibited the increasing level of active MMP-2 in cell extracts (Figure 2b).

Actinobacillus actinomycetemcomitans *supernatant down-regulated TIMP-2 in HPDL cells*

To determine whether the activation by *A. actinomycetemcomitans* supernatant was a result of the imbalance between MT1-MMP and TIMP-2, the levels of the proteins and gene expression were detected by Western blot and RT-PCR analysis, respectively. The results shown in Figure 4 demonstrated that the processes of MMP-2 activation by *A. actinomycetemcomitans* and *P. gingivalis* supernatant were different although the supernatant from both types of bacteria could exert MMP-2 activation.

In Western blot analysis (Figure 3a), the amount of MT1-MMP in the cultures treated with *A. actinomycetemcomitans* supernatant was not altered whereas its level was increased in the *P. gingivalis* supernatant treated cultures when compared with their control. On the contrary, TIMP-2 was decreased in the *A. actinomycetemcomitans* supernatant-treated cultures but not in those exposed to the *P. gingivalis* supernatant. A slight decrease in the level of TIMP-2 after treated with *P. gingivalis* was found in some experiments, however, the change was not statistically significant. Cell extract from HT1080 and culture medium of BT549 were used as positive control for MT1-MMP and TIMP-2 (Sato

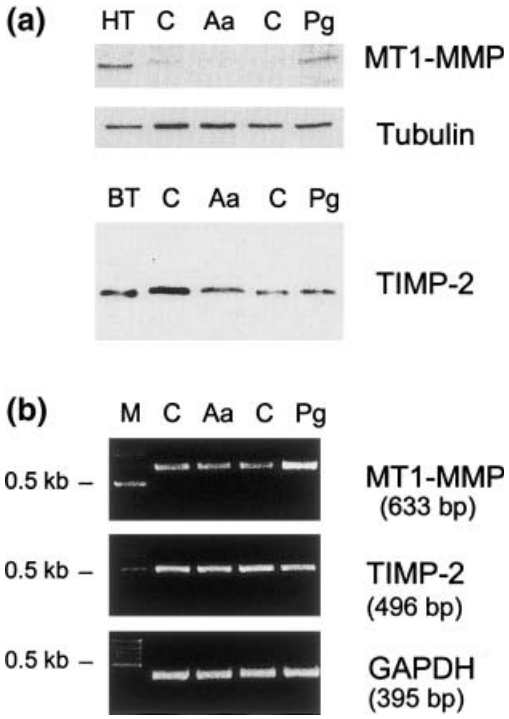

Figure 3 (**a**) The Western blot analysis of MT1-MMP and TIMP-2 after treatment with either *A. actinomycetemcomitans* (Aa) or *P. gingivalis* (Pg) supernatant. The results show that the Pg supernatant could up-regulate MT1-MMP in HPDL cells while the Aa supernatant down-regulated TIMP-2 when compared with its control. The level of tubulin was used as an internal control. Cell extracts from HT1080 (HT) and the medium from BT549 (BT) were used as positive controls for MT1-MMP and TIMP-2, respectively. (**b**) The RT-PCR analysis of MT1-MMP and TIMP-2. The results demonstrated that only the Pg supernatant could up-regulate the mRNA level of MT1-MMP in HPDL cells whereas the alteration of TIMP-2 was not observed. GAPDH was used as an internal control. M = DNA marker

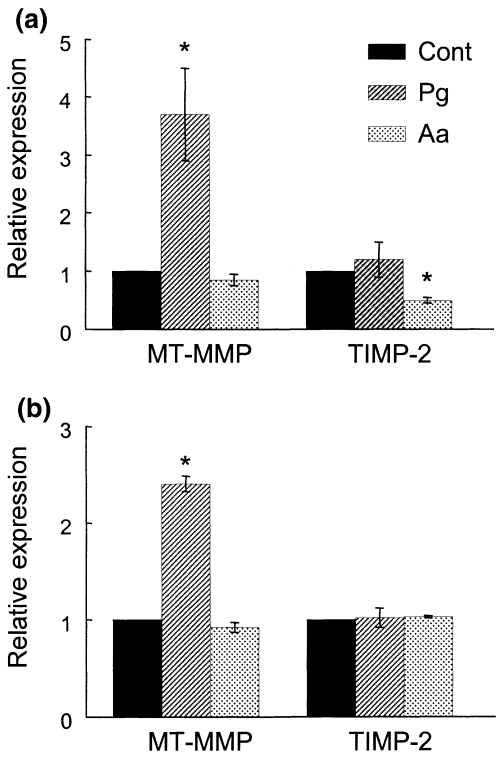

Figure 4 The histograms summarized the change of MT1-MMP and TIMP-2 from three different experiments. (**a**) The protein level, (**b**) the mRNA level of MT1-MMP and TIMP-2. The graph marked with asterisk denotes any significant differences when compared with its control (one-way ANOVA, Scheffe's test, $P < 0.05$). *Y*-axis represents the relative amount of expression. Pg = *P. gingivalis* supernatant, Aa = *A. actinomycetemcomitans* supernatant, Cont = control

et al, 1996; Gilles *et al*, 1998), respectively. The amounts of all proteins used were normalized to the levels of tubulin.

RT-PCR analysis in Figure 3b also exhibited different responses of HPDL cells to *A. actinomycetemcomitans* and *P. gingivalis* supernatant. The increased level of MT1-MMP was seen only in the cultures treated with the *P. gingivalis* supernatant when compared with the control. However, TIMP-2 expression alteration was not found in any of the cultures.

Figure 4 shows the summarization of MT1-MMP and TIMP-2 levels from three different experiments. In the culture treated with *P. gingivalis* supernatant, the protein level of MT1-MMP significantly increased about 3.5-fold over the control (Figure 4a) while the mRNA level increased about 2.5-fold (one-way ANOVA, Scheffe's test, $P < 0.05$) (Figure 4b). No significant change in the level of TIMP-2 was detected. When cells were treated with the supernatant from *A. actinomycetemcomitans*, only the protein level of TIMP-2 was significantly reduced onefold from the control ($P < 0.05$) (Figure 4a).

Discussion

The result from our previous report revealed that the *P. gingivalis* supernatant possessed the ability to activate MMP-2 secreted from HPDL cells (Pattamapun *et al*, 2003). In the present study, we proposed that this ability also persisted in the supernatant from *A. actinomycetemcomitans*. However, activation was not observed with *F. nucleatum*, another anaerobic bacterium found in periodontal pockets or with *E. coli* or *S. salivarius*, the other flora found in the oral cavity (data not shown). These findings suggested that the ability to activate MMP-2 is not a common characteristic of all bacteria in the oral cavity but is limited to some periodontopathogenic strains, such as *P. gingivalis* and *A. actinomycetemcomitans*.

In addition to the specificity of the bacterial supernatant in the activation of MMP-2, our results also indicate that the response to the supernatant is limited to certain cell types. In our previous work, we found that *P. gingivalis* supernatant could activate MMP-2 secreted from human gingival and pulpal fibroblasts (Pattamapun *et al*, 2003). In this study, both HPDL cells and HDF seemed responsible to the supernatant from *P. gingivalis* and *A. actinomycetemcomitans* in a similar fashion. However, U2OS, human osteosarcoma cell line, did not respond to the supernatant from *P. gingivalis* and *A. actinomycetemcomitans*. The results indicate that the response to the supernatant in terms of MMP-2 activation can be observed only in the fibroblasts or fibroblastic-like cells but not in bone cells. It is possible that this inductive ability of the supernatant might be limited to the cells that participate in soft tissue degradation.

It has been suggested that the association of active MMP-2 with cell surface proteins was important for ECM degradation (Brooks *et al*, 1996; Nakahara *et al*, 1997). To examine the presence of active MMP-2 on the cell surface, we also conducted gelatin zymography of cell extracts. We found an increased level of active MMP-2 on cell surface after treatment with the bacterial supernatant. This evidence implied that HPDL could directly use the active MMP-2 in the process of ECM degradation, leading to periodontal tissue destruction.

The diminished activation resulted by proteinase inhibitors suggested that *A. actinomycetemcomitans* as well as *P. gingivalis* supernatant could activate MMP-2 through an MMP-dependent mechanism. Although the effects attributed by *A. actinomycetemcomitans* and *P. gingivalis* supernatant were likely similar, the mechanism of activation was different. Activation by *A. actinomycetemcomitans* supernatant was correlated with reduction of TIMP-2 secretion without the alteration of MT1-MMP, while activation by *P. gingivalis* supernatant increased MT1-MMP but no change of TIMP-2 was found.

Our results are in concurrence with the MMP-2 activation model proposed by Strongin *et al* (1995) which demonstrated that the activation of MMP-2 required the balance of MT1-MMP and TIMP-2. The increased level of MT1-MMP as well as the decreased level of TIMP-2 could result in MMP-2 activation (Gilles *et al*, 1998; Ellerbroek and Stack, 1999; Sternlicht and Werb, 2001). The up-regulation of MT1-MMP by *P. gingivalis* supernatant reflected the availability of MT1-MMP on the cell surface leading to the MMP-2

activation. Whilst, the decreased level of TIMP-2 by *A. actinomycetemcomitans* may also reflect an increased level of MT1-MMP, which is not associated with TIMP-2. In general, molecules of MT1-MMP on the cell surface can be associated with TIMP-2 (Sternlicht and Werb, 2001). Only the excess MT1-MMP that is not associated with TIMP-2 can participate in the process of MMP-2 activation. Thus, the reduction of TIMP-2 by *A. actinomycetemcomitans* may result in an increase of excess MT1-MMP and consequently disturb the balance of MT1-MMP and TIMP-2, leading to MMP-2 activation. This result was also in concurrence with those reported by Gilles *et al* (1998) who showed that the reduction of TIMP-2 could induce MMP-2 activation in breast cancer cell lines.

It is interesting to note that the reduction of TIMP-2 by *A. actinomycetemcomitans* appears only at the protein level, as we did not observe any changes at the mRNA level. The reduction of TIMP-2 protein may be a result of the accelerated rate of TIMP-2 degradation by the secreted molecule(s) from the supernatant or of the direct degradation of TIMP-2 by the protease activity in the supernatant itself. The exact mechanism requires further investigation.

The virulence of the supernatant from *A. actinomycetemcomitans* and *P. gingivalis* on MMP-2 activation remains incomparable as the molecules that reside in the supernatant have not been elucidated. Further investigation is needed for the clarification of type of molecules and the different mechanisms of activation.

In conclusion, we found that both *A. actinomycetemcomitans* and *P. gingivalis* supernatant could activate MMP-2 in HPDL cells. This ability of MMP-2 activation was quite species-specific but not cell-type specific. Both supernatants from *A. actinomycetemcomitans* and *P. gingivalis* could induce the imbalance of MT1-MMP and TIMP-2 resulting in MMP-2 activation. These results indicate the importance of the balance between MT1-MMP and TIMP-2 in the pathogenesis of periodontal disease.

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