CHAPTER 2

ACTIVATION OF MMP-2 BY BACTERIAL SUPERNATANT CULTIVATED FROM PERIODONTAL POCKETS

2.1 Introduction

Human periodontal disease is associated with a complex microflora. Interactions between species as the plaque develops are important in pathogenesis of periodontal disease. The primary streptococcal colonizers of the tooth surfaces coaggregate with the secondary plaque-forming species, such as *Actinomyces* species (Kolenbrander et al., 1990). Further inter-species aggregation phenomena occur as the complexity of the maturating plaque. Gram-negative anaerobes colonize, such as *Veillonella* species followed by bacilli, including *Fusobacterium* and *Prevotella* species, which accumulate in succession, being followed in climax community by the spirochetes (Theilade, 1984a; Theilade, 1984b). Later in plaque maturation, the lectin-like interaction mediated coaggregation of *P. gingivalis* and *F. nucleatum* (Kinder and Holt, 1989). Thus, coaggregation between inter-species and adherence of oral microflora in the dynamic of dental plaque simulates human gingival sulcus as microenvironment for ecology of complex microflora. The multiple species of bacteria that have been implicated in the etiology of periodontitis may share a common characteristic or factor that is immediate cause of the disease. Under these conditions, any of several different species or combinations of species could account for disease.

Although, such variability can be attributed to differences in composition of the microflora in subgingival plaque but clinical data from previous study has been shown the composing of subgingival microflora, which most of the suspected pathogens are gram-negative anaerobes. The composition of viable bacteria was investigated paralleled with the disease activity (Liljenberg et al., 1994). No significant differences of total number of viable microorganism were observed between progressive disease active and progressive disease inactive lesion. These findings suggest that the multiple species of gram-negative anaerobic bacteria together with the presence of factors that might modify the host response to microbial assault, as well as factors that may predispose the individual to bacterial colonization at specific sites. Of these, it seems that the microflora composition and the host modifying factors are the most important regarding manifestation of periodontal disease. While the host response and environmental factors that affect this response are important for disease manifestation, gingivitis and periodontitis cannot commence without the presence of bacteria. A body of evidence suggests that bacteria are necessary for disease initiation, they are not sufficient to cause disease progression unless there is an associated inflammatory response (Offenbacher, 1996; Page and Beck, 1997). The destruction of connective tissue attachment of teeth and adjacent alveolar bone that observed in patients with chronic adult periodontitis is a consequence of host inflammatory reaction initiate in response to bacterial colonization of subgingival environment. Although the pathogenesis of various form of periodontal disease is not completely understood, host-derived proteases are believed to have an important role in disease progression. Previously, a number of studies have implicated matrix metalloproteinases (MMPs) and other proteases as playing a central role in a pathogenesis of the disease (Birkedal-Hansen, 1993).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are synthesized by a number of cell types in the periodontal tissues. Numerous studies have provided evidence for a degradative role of two subgroups of these enzymes, collagenases and gelatinases. The rationale for the important of collagenolytic activity is that collagens are the primary structural protein of the periodontium and their degradation is a central feature of progressive lesion (Page, 1995). Collagenases (MMP-1 from macrophage, epithelial cells and fibroblasts; MMP-8 from neutrophils) are Zn^{++} dependent endopeptidases, which act at physiological pH and temperature to cleave the native triple helix of the interstitial collagen at a single site in each polypeptide chain. In type I collagen, cleave occurs at the glycine-isoleucine bond in the alpha 1 (I) chain and at the glycine -leucine bond in the alpha 2 (I) chain about 3/4 along the molecule from the NH_2 end. Unlike bacteria enzymes, which make multiple cuts along the molecule, mammalian collagenases initiate degradation by making a single cut (Overall et al., 1987; Sorsa et al., 1988; Uitto et al., 2000). Subsequent degradation of denature collagen molecule can be degraded by fibroblast-type gelatinase (MMP-2 or gelatinase A) or epithelial-type gelatinase (MMP-9 or gelatinase B).

Although, several studies strongly suggest that MMP1, MMP-8, MMP-9 and recently MMP-2 (Birkedal-Hansen, 1993; Komatsu et al., 2001) associated in periodontal tissue destruction. MMP-2 was found in the gingival crevicular fluid of periodontitis patients and in the gingival tissue of the patients (Makela et al., 1994). In addition, the increasing amount of active MMP-2 that localized within periodontal lesion only was recently shown in chronic adult periodontitis (Korostoff et al., 2000).

These results suggest the involvement of MMP-2 in periodontal tissue destruction. Many reports have demonstrated the prevalence of various bacterial species around the tooth surface of periodontitis associated with the expression profiles of MMP-1 MMP-8 (DeCarlo et al., 1997; Sorsa et al., 1988) but there was a little study investigates the effects of multiple species of gram-negative anaerobic bacteria. The combination of bacterial products from inter-playing complex microflora may enhance the virulence of themselves in periodontal tissue destruction. There was a few studies determine the responses of host cell, which locally face to bacteria and their products accumulated in periodontal pockets. Thus, human gingival fibroblasts (HGF) and human periodontal ligament (HPDL) cells were used to direct demonstrate the effect of bacterial products on host-derived enzymes, particularly MMP-2 which constitutive produced and locally secreted by these cells in periodontal tissue. And also, the mechanism by which bacterial product regulates MMP-2 expression and MMP-2 function in HGF and HPDL cells has not been elucidated.

The aim of this study was to determine the effect of bacterial products from multiple species of gram-negative anaerobes, which cultivated from periodontal pockets on MMP-2 activation. Using human periodontal ligament (HPDL) cells and human gingival fibroblasts (HGF) in cultured stimulated by bacterial supernatant. Analyzed the enzyme activity of MMP-2 by mean of zymography.

2.2 Materials and Methods

Bacterial culture

Subjects who had been referred to the Department of Periodontology Chulalongkorn University were selected to participate in this study and gave informed consent to our experimental procedure. The patients were clinically diagnosed as adult periodontitis, free of systemic disease and did not receive any periodontal or antibiotic therapy for 6 months prior to this experimental procedure. Gingival crevicular fluid (GCF) were collected using sterile fine paper points (Johnson and Johnson, East Windsor, United Kingdom) according to the method mentioned in the previous study (Condorelli et al., 1998). Briefly, the area of the collection site was isolated with cotton roll and dried with air. After subgingival plaque was carefully removed, the sterile paper point was insert to periodontal pocket until mild resistance was felt and held for 10 seconds. Collected GCF samples in reduced transport fluid (RTF), stored at $-80\ ^{O}C$ until use.

Bacterial supernatant preparation

Bacteria in RTF medium were inoculated into Tryptic soy broth (TSB; BBL Microbiology Systems, Cockeysville, MD) incubate at $37\ ^{o}C$ in anaerobic jars and generate the appropriated anaerobic condition by using Gas Pak® (BBL Microbiology Systems, Cockeysville, Md.) for 5-7 days, or until an absorbance of bacteria reached 1.000 at 660 nm. Bacterial broth was centrifuged at 6000xg, $4\ ^{O}C$, 15 min and the

supernatant was collected. The supernatant was filtered twice through a 0.22 μm membrane (Gelman Science, USA), stored at –80 ^{O}C until use.

Cell culture

HPDL cells were cultured from explants obtained from non-carious, freshly extracted third molars for orthodontic reason. All patients gave informed consent. Teeth were rinsed with sterile phosphate buffer saline several times and the PDL tissues were scraped out from the middle third of the root as previously described (Ragnarsson *et al.*, 1985. The explants were harvested on a 60-mm culture dish (Nunc, Roskilde, Denmark) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-Glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B at 37 ^{O}C in humidified atmosphere of 95% air, 5% CO_2. Medium and the supplement were from Gibco BRL (Gibco Laboratories., Grand Island, NY). The medium was replaced every other day until cells grew out from the explants. After reaching confluence, cells were subcultured with a 1:3 ratio. For this study, cells from third to fifth passages were used.

Human gingival fibroblasts (HGF) were prepared from the explants of human gingiva obtained from the extracted molars. Fibroblasts were isolated and cultured as described above.

Activation of HPDL and HGF cells with bacterial supernatant

HPDL cells were seeded in 24-well plates (Nunc) at a density of 50,000 cells/ml/well (25,000 cells/cm^2) and allowed to attach for 16 hours. Cells were silenced overnight in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO) before the treatment. Cells were treated with various concentrations of supernatant in serum-free condition. The equal amount of TSB was also added to the control.

Next, HGF cells were seeded and silenced as described above. Various concentrations of supernatant that do not show any toxicity were added into the culture medium. After 48 hours, the medium was collected and kept at -20°C prior to the MMP-2 analysis.

In the inhibitory experiment, cells were treated with inhibitors for 30 minutes before supernatant was added. Using 2 mM ethylene diamine tetra-acetic acid (EDTA) from Sigma was used as inhibitor in this experiment.

MTT assays

MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells to a purple formazan product that is insoluble in aqueous solution. The end point of assay was an estimate of cell number by indirect method. MTT is widely uses not only for a viability assays but also for a cytotoxicity assay. The assay was performed as mention in Freshney (1994). Briefly, cells were seeded at 50000 cells/ml/well in 24-well plate for 24 hours and incubated in serum-free medium for 6 hours. Cells

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were then treated with or without bacterial supernatant at 1:5, 1:25, 1:10, and 1:100 in serum-free medium for another 24 hours. The medium was changed to serum-free medium without phenol red containing 5mg/ml MTT for the last 3 hours of incubation. After removing the medium from each well, the formazan reaction products of MTT was dissolved in 900 μl DMSO (Sigma) and 125 μl glycine buffer. The reading was done by mean of spectrophotometry (Ultraspec 3000, Pharmacia Biotech, Piscataway, NJ.) at 570 nm. Cell numbers were determined by comparision to the standard curve which creating from the known cell number and convert into a percentage. The absorbance of dissolved-MTT formazan reaction product from the wells which have no cell were use to be blank.

Statistical analysis

The data were expressed as percent mean ± standard deviation of triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Scheffe test. A *P*-value of < 0.05 was considered statistically significant.

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 non-reducing condition. Equal amount of protein, measured by BCA™ protein assay (Pierce, Rockford, IL), was loaded in each lane. After electrophoresis, the gel was gently shaken in denaturing buffer (0.25% Triton-X100) at room temperature for 30

minutes to remove SDS, and then incubated in developing buffer (0.15M NaCl/10 mM $CaCl_2$/50mM Tris-HCl pH 7.5/0.1% Brij-35) at 37°C for 20 hours. The gel was stained with 2.5% Coomassie brilliant blue-R in 30% methanol and 10% acetic acid. Latent and active MMP-2 can be detected as the clear bands at the position of 72 kDa and 62 kDa, respectively.

2.3 Results

In this study, MTT assay was used to analyze cytotoxicity of cultured cells as shown in **Figure 2.1**. The graph shows the toxicity of each dilution of bacterial supernatant. Cells were grown and treated as described above for 24 hours. Cytotoxicity was measured by MTT assay. The OD was converted into cell number by comparing with the standard curve. The result indicates that the cell number is not affected by any concentrations of bacterial supernatant except at the concentration 50 μl/ml, which the cell numbers, decreases approximately 20 %.

Next, we investigated the effect of bacterial products at non-toxic dose on MMP-2 activation. After HPDL were seeded in 24 well plate and treated with bacterial supernatant at the concentration of bacterial supernatant in culture medium were 0, 5, 10, 15, and 20 μl/ml for 48 hours. Our results revealed that at non-toxic doses of bacterial products (5-20 μl/ml) could activate MMP-2 as shown in **figure 2.2**, MMP-2 activation was detected in lane 2-5, whereas control (lane 1) could not reveal the active band of MMP-2. MMP-2 activation was examined by gelatin zymography. The clear bands appeared at 72 kDa and 62 kDa correspond to the latent and active MMP-2, respectively. The results indicate that bacterial supernatant can

activate MMP-2 in a dose dependent manner when compared with control (c). Similar result was also found in HGF experiment (data not shown).

In order to examine the mechanism of MMP-2 activation by mixed anaerobe supernatant Cells were preincubated with 2 mM EDTA, a chelating agent, for 30 minutes and then stimulated with bacterial supernatant at the concentration of 10 μ l/ml for 48 hours. The culture medium was subjected to analyzed MMP-2 activity by mean of gelatin zymography. It was found that bacterial–induced MMP-2 activation was completely inhibit in the presence of EDTA as shown in **Figure 2.3.** To determine the pattern of bacterial supernatant-induced MMP-2 activation in different cases which cultivated from different individual periodontal pocket's patients. Our observation was shown in **figure 2.4.** In this study bacterial supernatant was prepared from 3 different patients and was used to activate cultured cells at 10 μl/ml for 48 hours. The results from gelatin zymography clearly demonstrated that all preparations of bacterial supernatant could similarly activate MMP-2.

2.4 Discussion

The bacterial etiology in periodontal pocket is complex with a variety of organisms responsible for the initiation and progression of disease. In this study, I have undertaken a model of cell culture to directly demonstrate the involvement of bacterial products releasing from mixed-bacterial population in MMP-2 activation. Thus, the first part of this experimenta involved the preparation of bacterial supernatant from mixed bacterial samples colonized in periodontal pockets. HGF and HPDL were stimulated by bacterial supernatant for 48 hours. MMP-2 activation in

cultured medium was analyzed by zymography. Like other member of MMP family, MMP-2 is secreted as a latent form (proenzyme) and its function needs activation process, which is the most important step for substrate degradation. Zymogram was use to determine the secreted MMP-2 that may be activated by bacterial products of mixed gram-negative anaerobic bacteria. This method provides a useful mean of detecting both the precursor and active forms of MMP-2 in the same gel which reveals two clear bands against dark-blue background. After electrophoresis, washing the gel with a buffer at a neutral pH containing a non-ionic detergent with a small amount of Ca^{++}, this step renature the proenzyme. After incubating the gelatin-gel at 37°C for 18 hours, the proenzyme that is activated has ability to digests the gelatin in the gel. Thus, proMMP-2 and or active MMP-2 migrates to position corresponding to the molecular mass of 72 kDa and 62 kDa indicate migration band of proMMP-2 and active form of MMP-2, respectively. It was found that treatment of HGF and HPDL cells with culture supernatant of mixed anaerobes resulted in the production of active MMP-2 in fully active form of MMP-2 with a molecular mass of 62 kDa, whereas non-treated control cultures did not show the active form.

Generally, colonized-bacteria in periodontal pocket utilizes nutrient sources from host and during their growing they produced and secreted not only metabolic products but also various kind of bacterial products such as proteolytic enzymes, membrane blebs, which may enhance the virulence of localized mixed-infection. The virulence of some species, such as black-pigmented gram-negative anaerobes, could produce a powerful proteolytic enzyme, which directly degrade host cells and also host proteins. Moreover, other virulence factors secreting from these gram-negative bacteria have a high potential toxicity including LPS, volatile sulfur compound such

as hydrogen sulfide, methylmercaptan, dimethyl disulfide, which are cytotoxic and can inhibit protein synthesis (Lamont and Jenkinson, 1998). The results also shown that at the concentration of 50 µl/ml of supeınatant of mixed anaerobes, which enrich of bacterial products from multiple species of bacteria cultivated from periodontal pocket, have a potent cytotoxicity to host cells by directly degrades host cell surface protein. Examination under phase contrast, cell morphology of cultured cells were round up and some of them could not adhere to the culture plate. In addition, the bacterial products at non-toxic level could induce MMP-2 activation and this process could be inhibited by the presence of EDTA. The reason for the inhibition effect of EDTA is the results from its metal chelating agent that can competitively bind to the metal ion (Zn^{++} or Ca^{++}) in the system. From previous study, MMP-2 activation required the function of another MMP, namely MT1-MMP (Azzam and Thompson, 1992; Strongin et al., 1995). Thus, the presence of EDTA led to MT1-MMP dysfunction, then the latent MMP-2 could not be processed to active form. These results suggested that low amount of bacterial products acted as triggering molecules and regulated host enzymes to function. As we try to proof the capability of bacterial supernatant-induced MMP-2 activation via cell-independent pathway. It was found that bacterial supernatant could not induce MMP-2 activation in condition medium (data not shown). From these results, MMP-2 activation in the presence of mixed anaerobe supernatant depended on another enzyme function, which is metalloenzyme.

Although human periodontal disease is associated with a complex microflora, it has been shown that the most significant bacteria associated with periodontitis are *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis,* and *Bacteroides forsythus* (Zambon, 1996). Pathogenic bacteria are known to colonize by variety of

highly specific mechanism. The mechanism by which one bacteria-species adheres to another or to host tissue cells. In addition to its adherence activity, the periopathogenicity of bacterium, which is predominant at a certain site, may have a specialized capability to colonize and secrete bacterial products for serving their growth. *Porphyromonas gingivalis* is one of the most putative periodontopathogenic bacteria. *P. gingivalis* is well equipped with a wide array of structural and functional features which enable it to colonize either the gingival sulcus or periodontal pockets. This organism could survive in this microenvironment of host by successfully evading host antimicrobial defenses. They also utilized such putative virulence factors as fimbriae and lectin-type adhesions, a polysaccharide capsule and lipopolysaccharide, hemagglutinating and hemolysin, release of toxic products of metabolism, outer membrane vesicles and numerous enzymes, all of which have been extensive reviewed (Mayrand and Holt, 1988; Sundqvist et al., 1988; Smalley and Birss, 1991).

For future direction, it is necessary to study the pathogenic mechanism of *P. gingivalis* in periodontal tissue destruction. In term of the effects of bacterial products released from *Porphyromonas gingivalis* and MMP-2 activation whether it could induced MMP-2 activation via transcriptional or non-transcriptional pathway. The remaining research questions to be answer is *P. gingivalis* products affect on MMP-2 activation and expression profiles of MMP-2, MT1-MMP or TIMP-2 or not. Previously, the studies of some bacterial species which associated periodontal disease were reported including *Eikenella corrodens* stimulated 3-fold the production of mRNA encoding MMP-2 while other MMPs mRNA remained unchanged (Dahan et al., 2001). In addition, a novel oral spirochete species; *Treponema lecithinolyticum* in human GF and PDL cells demonstrated that whole cell sonicated, outer membrane

fraction and formaldehyde-fixed cells of *T. lecithinolyticum* can induce MMP-2 activation (Choi et al., 2001). These studies suggested that each bacterial species posses abilities to participate in extracellular matrix degradation by activation of host-derived MMP-2 during periodontal inflammation

Conclusion

Bacterial supernatant which enrich of numerous bacterial products from multiple species of gram-negative bacteria cultivated from periodontal pockets, at non-toxic level could induce MMP-2 activation in both HPDL and HGF cells. This process could be inhibited in the presence of EDTA.

Figure 2.1 Cytotoxicity of mixed anaerobes supernatant Toxicity of mixed anaerobe supernatant determined by MTT assay cell were treated with 0, 5, 10, 15, 20, 50 μl/ ml of supernatant for 24 hours. Data were shown in mean ± SD from three independent experiments. Toxicity of supernatant was observed at 50 μl/ ml. Statistical analysis was performed by one way analysis of variance (ANOVA) using Scheffe test. A *p*-value of < 0.05 was considered statistically significant.(*).

Figure 2.2 Dose relationship of mixed anaerobes supernatant on MMP-2 activation MMP-2 activation in HPDL cells were response in dose dependent relationship. Cells were grown in the absence (control)or presence of various concentration of mixed anaerobe supernatant (0, 5, 10, 15, 20 μl/ml) for 48 hours. The media were collected for gelatin zymography. The position of latent and active MMP-2 were indicated on the right.

Figure 2.1 Cytotoxicity of mixed anaerobes supernatant

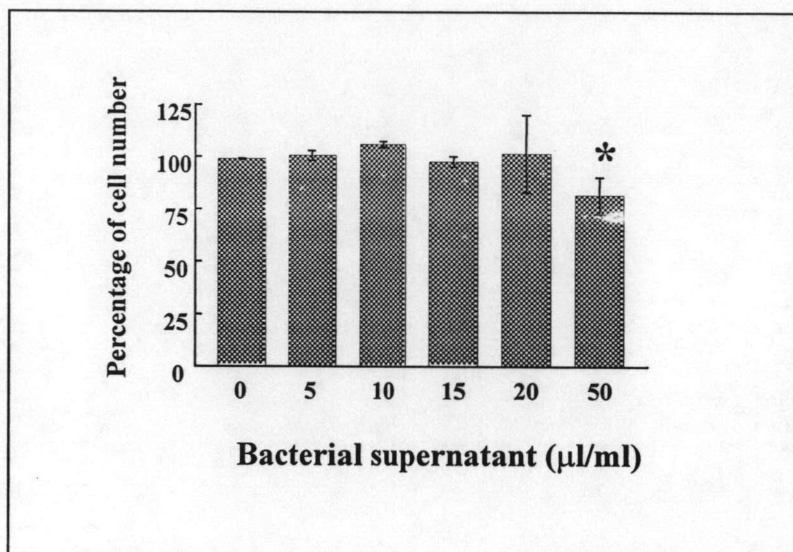

Figure 2.2 Dose relationship of mixed anaerobes supernatant on MMP-2 activation

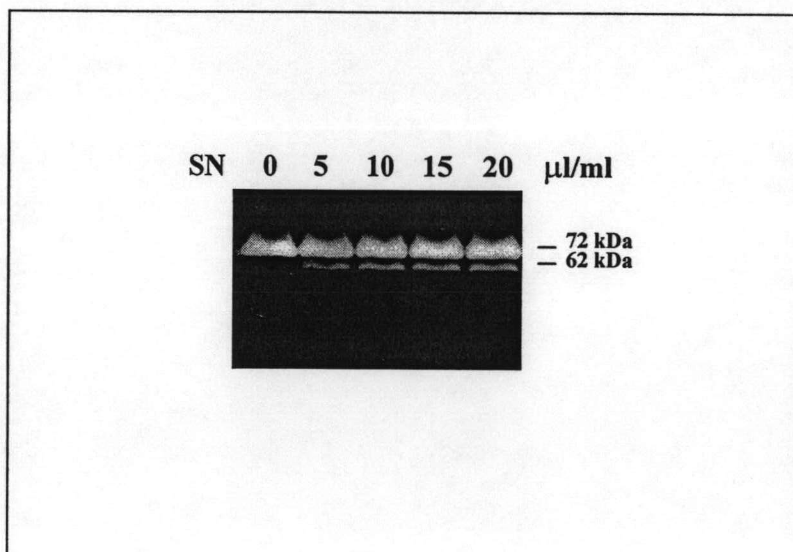

Figure 2.3 Effect of metal chelating agent (EDTA) on MMP-2 activation
In the inhibitory experiment, cells were treated with proteinase inhibitors for 30 minutes before mixed anaerobes supernatant (10 μl/ml) was added. Metal chelating agents; 2 mM Ethylene diamine tetra-acetic acid (EDTA) was used to . inhibit MMP-2 activation process. Condition medium of each was collected and determined MMP-2 activation by gelatin zymography.

Figure 2.4 Effect of supernatant of bacteria sampling from three patients on MMP-2 activation. Culture cells were treated with mixed anaerobes supernatant obtained from three different patients. Latent and activate MMP-2 (72 kDa and 62 kDa) were indicated in the right. MMP-2 activation was observed in treated experiment. Control, cells were treated with equal amount of tryptic soy broth (TSB).

Figure 2.3 Effect of metal chelating agent (EDTA) on MMP-2 activation

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EDTA - - +

Figure 2.4 Effect of supernatant of bacteria sampling from three patients on MMP-2 activation

1 2 3

SN - + - + - +

— 72 kDa
— 62 kDa