CHAPTER 4

THE EFFECT OF *Porphyromonas gingivalis* LIPOPOLYSACCHARIDE ON MMP-2 ACTIVATION

4.1 Introduction

Periodontal disease is the major cause of adult tooth loss and is commonly characterized by a chronic inflammation caused by infection of oral bacteria. *Porphyromonas gingivalis (P. gingivalis)* is one of the suspected periodontopathic bacteria and is frequently isolated from the periodontal pockets of patients with chronic periodontal disease (Socransky et al., 1998). Numerous clinical studies have been reported that *P. gingivalis* associated with periodontal tissue destruction. The presence of *P. gingivalis* in destructive periodontitis, with increase prevalence and proportion in progressive compared with quiescent sites of the disease (Curtis et al., 1991; Haffajee and Socransky, 2000). *P. gingivalis* exhibits a variety of virulence factors that are believed to essential for its ability to colonize and promote destruction (Chen et al., 1991). *P. gingivalis*, asaccharolytic, a highly proteolytic gram-negative bacteria, utilized the host protein as nutrient sources for their growth by secreting various kinds of proteases and also a large amount of vesicle (Slots and Listgerten, 1988). Like other gram-negative bacteria, *P. gingivalis* has been demonstrated to elaborate vesicles, which is constitutively secreted from membrane blebs. These relatively small structures contain many of putative virulence factors of these organisms; adhesin, proteases and lipopolysaccharide (LPS) (Grenier and McBride, 1989). There is now increasing evidence for the importance of LPS in a crucial role of

alveolar bone destruction (Gessani et al., 1993; Hayashi et a., 1996; Wang et al., 2000; Lorenz et al., 2002)

LPS is a major component of the outer membrane of gram-negative bacteria. Unlike most types of bacteria, gram-negative bacteria surround themselves with a double membrane. The outermost of these two membranes is asymmetric, with the inner monolayer composed of glycerophospholipids and the outer monolayer composed mostly of lipopolysaccharide. LPS is sometimes called endotoxin because of its toxic, inflammatory properties, which can result in a potentially fatal phenomenon known as septic shock (Stone, 1994). A major function of this outer membrane is widely believed to serve as a protective barrier. The outer membrane make the bacteria resistant to a variety of host defense factors, such as β-lysin, lysozyme, and various leukocyte proteins, which are quite toxic to gram-positive bacteria (Donalson et al., 1974; Patterson-Delafield et al., 1980). Furthermore, the outer membrane of gram-negative bacteria acts as a barrier to the diffusion of many antibiotics (Hancock, 1984). LPS is released during growth or lysis of bacteria and acts as a potent inflammatory stimulus, eliciting a range of host responses such as cytokine and growth factor releasing, inflammatory mediator in periodontal disease (Okada and Murakami, 1998). Previously, quite a lot of studies examine the effect of LPS on cellular response, such as LPS of *E. coli* have shown to enhance the production of cytokines during differentiation of human monocytes to macrophages (Gessani et al., 1993), induce respiratory inflammation in mice (Isaac et al., 1999).

Inflammatory response by host is the major pathway of connective tissue destruction especially host derived enzymes, matrix metalloproteinases (MMPs)

which is a group of endopeptidases that plays role in the turnover of connective tissue in physiological and pathological conditions, including periodontal disease. The members of MMP family that have been implicated in periodontal tissue destruction involve MMP-1, MMP-8, MMP-9 and recently MMP-2. The presence of MMP-2 and the increase of active MMP-2 in periodontal lesion were recently shown to associate with periodontal destruction in chronic adult periodontitis. (Makela et al., 1994; Korostoff et al., 2000) MMP-2 or 72-kDa type IV collagenase has been demonstrated to play an important role in turnover or degradation of soft connective tissue. MMP-2 can degrade type IV collagen, the major protein found in the basement membrane. Moreover, recent evidences revealed that this enzyme can also degrade type I collagen, which is the major protein in periodontal tissue (Tournier et al., 1994; Aimes and Quigley, 1995). The correlation between *P. gingivalis* products and MMP secretion has been described (Uitto et al., 1989; Sorsa et al., 1992; Fravalo et al., 1996; Decarlo et al., 1997; Firth et al., 1997). The products that have been shown to contain MMP induction ability include the outer membrane extract and the bacterial phospholipase C, proteinases secreted from *P. gingivalis* contain the inductive ability and the activation ability for enzymatic function of MMPs. (Ding et al., 1995; Potempa et al., 1995). There was no study focusing on the ability of LPS induced MMP-2 activation, particularly the effect of *P. gingivalis* LPS.

As mention above, *P. gingivalis* is predominant in periodontal pockets. This species of bacteria has been demonstrated to elaborate vesicles and secreted from membrane blebs, composed of structural components similar to its outer membrane of its such as, proteases and LPS. LPS is considered to be a major factor in the pathogenesis of periodontal disease, and it is absorbed into the root surface and

gingival tissue of patients with periodontal disease (Mayrand and Holt, 1986). Removing endotoxin at root surface by root planning promoted a better periodontal tissue healing (Jones and O'Leary, 1978). LPS molecules may involve in cellular response of local host cells, causing the dysregulation of host derived enzyme, especially MMP-2.

ProMMP-2 requires further activation process to be fully active form before its function. An imbalance of enzyme regulation resulting in the accelerated break down of periodontal connective tissue. The purpose of this study was to determine the effects of *P. gingivalis* LPS on MMP-2 activation in human periodontal ligament (HPDL) cells and human gingival fibroblasts (HGF). Fibroblasts and also epithelial cell were previously considered as primarily cells that provide a physical barriers and structural components in periodontium. Accordingly, it is now recognized that these local cells also play a crucial role in periodontal destruction. In periodontitis, human periodontal ligament (HPDL) cells and gingival fibroblasts (HGF) are a major group of cells highly affected by the *P. gingivalis* products. Although they constitutively synthesize and secrete MMP-2 but MMP-2 activation was tight regulated. The results in Chapter 3 have already shown that bacterial products from *P. gingivalis* could activate proMMP-2 to be active MMP-2. On the other hand bacterial products from *P. gingivalis* have no effect on MMP-2 expression both transcriptional and translational levels. To date, there is no study focusing on the effect of *P. gingivalis* LPS on MMP-2 activation in human primary cell culture; HGF and HPDL cells. Since fibroblasts, which are the major constituents of gingival connective tissue and periodontal tissue, may directly interact with bacteria and bacterial products, particularly LPS, in periodontitis lesions. The objective of this study was to investigate the correlation

between *P. gingivalis* LPS and MMP-2 activation in HPDL and HGF. Both cell types were established and used to direct demonstrate the response of human fibroblasts to *P. gingivalis* LPS.

4.2 Materials and Methods

Cell culture

HPDL and HGF cells were cultured from explants obtained from freshly extracted third molars for orthodontic reason. The detail was mentioned in materials and methods part of Chapter 2.

Lipopolysaccharide isolation

P. gingivalis from periodontal pocket was prepared as described in chapter 3. All strains of bacteria were cultured in TSB supplemented with 5 μg/ml hemin and 1 μg/ml menadione (as describe in Chapter 3). *P. gingivalis* were cultured for 5 days. After harvesting, bacteria were sedimented in microcentrifuge. LPS was prepared by the rapid method as described by Al-Hendy et al. (1991) and the procedure was shown in **figure 4.1.** A 1.5-ml of cultured bacteria was centrifuge at 16000xg for 30 sec. The bacterial pellet was suspended in 100 μl of TAE buffer (40 mM Tris-acetate, pH 8.5-2 mM EDTA) and mixed with 200 μl of alkaline solution (3 g SDS, 0.6 g Trizma base (Sigma), and 6.4 ml 2 N NaOH in 100 ml H_2O). The mixture was heated at 60 °C for 70 min, mixed with preheated phenol-chloroform (1:1, v/v) and incubated at 60 °C for another 15 minutes. After centrifugation at 16000xg for 10 min, the

supernatant was collected and digested with proteinase K (Boehrringer Mannheim GmBH, Germany) at 60 °C for 60 min. The solution was mixed with 200 µl H_2O and 50 µl of 3 M sodium acetate (pH 5.2). The LPS was precipitated by adding 2 volumes of ethanol.

In order to confirm the presence of LPS from the rapid method, two addition tests were conducted: silver stain to analyze the ladder pattern of LPS and determine the endotoxin activity by comparing our isolated LPS and commercial LPS from *E. coli* using E-TOXATE (Sigma Chemical Co., St. Louise, MO).

Silver staining

LPS was subjected to electrophoresis, using a method described by Tsai and Frasch (1982) with a slight modification. Briefly, the LPS samples were mixed with an equal volume of 0.1 M Tris-HCl buffer, pH 6.8 containing 2% (w/v) SDS, 20% sucrose (w/v), 1% DTT (v/v), and 0.001% bromophenol blue (w/v). The mixture was heated in a 100 °C water bath for 5 min, the samples were applied to sample well and subjected to electrophoresis in a SDS-gel electrophoresis system using 12% separating gel at 20 mA/ slab gel until the bromophenol blue migrated 10 cm. LPS in the gel were then visualized by the non-diamine method silver staining as described in Merril et al.(1981). This stain method is recommended for gels less than 1 mm in thickness. A step of staining was done in glass tray, place the tray on orbital shaker at a very low speed and do all steps at room temperature. In brief, the gel was fixed in fixation solution (50% methanol, 10% acetic acid) for 1 hour or overnight if necessary. The gel was soaked in rehydration solution (10% methanol, 5% aceic acid)

for 10 min, followed by a 2% glutaldehyde solution for 30 min. Gel was washed with deionized water for 15 min, before soking the gel in 0.1% potassium dichromate solution. Next, gel was incubated in a 0.2% silver nitrate solution for 25 min, and washed with 50 ml of sodium carbonate/formaldehyde solution. The washing was stopped as soon as the gel turned gray with colloidal particles. To stop the image development, discard the developing solution and then add 250 ml of 3% acetic acid solution. The gel was washed two times with 250 ml of deionized water for 10 min each wash. The gels were stored in moisture condition and covered with the clear plastic bags.

Endotoxin activity

The extracted LPS (*P. gingivalis* LPS) was subjected to determine the endotoxin activity by using the *Limulus* amebocyte lysate (LAL) test. The procedure of endotoxin activity determination was done according to manufacturer's instruction. Briefly, all LPS dilutions were prepared in sterile, capped polystyrene tubes. The standard LPS was diluted in endotoxin free water to give the concentration of LPS ranging from 0.015 - 400 EU/ml. The lyophilized *Limulus* amebocyte lysate were reconstituted with endotoxin-free water. Added E-TOXATE working solution to each tube by inserting pipette to just above the contents and allowing lysate to flow down the side of tube, thereby avoiding contact and possible contamination. The addition of lysate to tube containing least (expected) endotoxin first, i.e., tube C followed by tube A, then lowest through highest positive standards and finally tube B, for reducing possible contamination. Tube contents were mixed gently. The tubes were covered

with foil or parafilm and incubated for 1 hour undisturbed at 37 °C. Reading the results after 1-hour incubation.

All assays using multiple vials performed in 10x75 mm glass culture tubes. The tubes were covered with small squares of foil or parafilm during incubation.

Endotoxin (EU/ml) =(1/the inverse of the highest dilution of sample) x Final concentration of endotoxin standard dilution which has a positive result (Appearance of hardening gel)

MMP-2 activation by *P. gingivalis* LPS

Gingival fibroblasts were seeded in 24-well plates (Nunc, Roskilde, Denmark) at a density of 50000 cells/ ml/ well and allowed to attach for 16 hours. Cells were silenced overnight in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma) before the treatment. Cells were treated with various concentration of *P. gingivalis* LPS in serum-free condition. The equal amount of sterile-distilled water was also added to control.

Next, human periodontal ligament cells were seeded and silenced as described above. Various concentrations of *P. gingivalis* LPS (0-20 μg/ml) that did 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 activation analysis.

In the inhibitory experiment, cells were treated with either proteinase inhibitors or NF-kB inhibitor for 30 minutes before *P. gingivalis* LPS was added. Proteinase inhibitors used in the experiment included 10 μM phenanthroline, 2 mM Ethylene diamine tetra-acetic acid (EDTA), 10 μM phenanthroline, 55 ng/ml (0.25 Trypsin inhibitor unit; TIU) aprotinin. Cells were preincubated with 50 μg/ml NF-kB inhibitor, APDC, for 30 minutes before adding *P. gingivalis* supernatant. All inhibitors were obtained from Sigma.

Direct activation of MMP-2 by *P. gingivalis* LPS

Cells (HPDL/HGF) were seeded in 24-well plates (Nunc) at a density of 50000-cells/ ml/ well and allowed to attach for 16 hours. Cells were silenced in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma) for 48 hours. The culture medium was collected and will be called the condition medium. The 500 μl volume of conditioned medium was added with 10 μg/ml of *P. gingivalis* LPS in the presence or absence of 10 μM phenanthroline, 55 ng/ml (0.25 Trypsin inhibitor unit; TIU) aprotinin, 0.2 mM phenylmethylsulfonylfluoride (PMSF) for 24 hours at 37 °C. Another experiment, the conditioned medium of cultured cells was incubated with heat treatment of *P. gingivalis* LPS for 24 hours. At the end of the experiments, a portion (10 μl) of the incubation mixture was subjected to MMP-2 activation analysis by gelatin zymography as describe in chapter 2.

4.3 Results

Endotoxin activity

Figure 4.1 summarized the rapid method, which was used for LPS extraction from both reference and clinical isolated strains of *P. gingivalis.* In order to ensure the LPS preparation technique, the endotoxin activity of LPS was determined by using the *Limulus* amebocyte lysate test (E-TOXATE kit) in all LPS preparations. The endotoxin activity is characterized by coagulating ability in the presence of *Limulus* amebocyte lysate. **Table 4.1** shows the coagulating ability of each LPS-dilution in the presence of *Limulus* amebocyte lysate. *P. gingivalis* LPS from both reference and clinical isolated strains reveal the gel hardening at the concentration equivalent to *E. coli* endotoxin unit at 0.125 EU/ml (Tube D3). These interpretations give the calculation results of endotoxin unit at 4 EU/ml.

Silver staining

The presence of LPS was further confirmed using SDS-PAGE and silver stain technique. The ladder pattern of LPS, which is the character of gram-negative bacteria LPS, was found as shown in **Figure 4.2**. Lane 1, LPS of *P. gingivalis* (W50); lane 2 and 3, LPS of clinical isolated *P. gingivalis* from patient-1 and patient-3, respectively. An ultra sensitive silver-based color stain for LPS in polyacrylamide gels showed many orderly space band represent LPS molecules having different numbers of repeating units in their O side chains. The fastest migration band was the LPS having only a complete core; the second fastest band was the core plus one repeating unit.

MMP-2 activation by *P. gingivalis* LPS

The toxicity of LPS was investigated using MTT assay. Various dilutions of LPS from 0-100 μg/ml were tested. The results were shown in **Figure 4.3 A.** The toxicity was found when using more than or equal to 40 μg/ml of LPS. The LPS concentrations ranging from 0-20 μg/ml, which have no cytotoxic effect on cultured cells, were selected to activate the culture cells. The conditioned medium of cultured cells was subject to analyze MMP-2 activation by zymography. The extracted LPS could induce the activation of proMMP-2 in human PDL fibroblast and gingival fibroblast in dose response relationship manner as shown in **Figure 4.3 B.**

Inhibitory effect of protease inhibitors on MMP-2 activation by *P. gingivalis* LPS

The next experiments, various proteinase inhibitors were added into the culture medium for 30 minutes prior to the application of *P. gingivalis* LPS. The results in **Figure 4.4A** indicated that heat-denatured LPS could not activate MMP-2. EDTA and phenanthroline had no inhibitory effect on LPS induced MMP-2 activation. However, serine proteinase inhibitors (aprotinin) had the inhibitory effect on LPS induced MMP-2 activation. All protease inhibitor had no toxicity effect as shown in **Figure 4.4B**.

Effect of NF-kB inhibitor on MMP-2 activation by *P. gingivalis* LPS

Cells were preincubated with 50 μg/ml of NF-kB inhibitor (APDC) for 30 min before stimulating with *P. gingivalis* LPS for 48 hours. The conditioned medium of cultured cells was subjected to analyze MMP-2 activation by gelatin zymography. It

was found that in the presence of NF-kB inhibitor MMP-2 activation was partially inhibited as shown in **Figure 4.5.**

Direct activation of MMP-2 by *P. gingivalis* LPS

From the above results, MMP-2 activation in the presence of APDC was partially inhibited. These results indicated that MMP-2 activation was partial regulated via NF-kB dependent pathway. Therefore, we hypothesized that MMP-2 activation possibly directly process by *P. gingivalis* LPS.

The conditioned medium of culture cells was incubated with *P. gingivalis* LPS in the presence or absence of protease inhibitor for 24 hours. Another experiment, conditioned medium of culture cells was incubated with heat treatment LPS for 24 hours. It was found that *P. gingivalis* LPS could directly activated MMP-2. This process was inhibited by a presenting of serine protease inhibitor (aprotinin), whereas phenanthroline and EDTA had no inhibitory effect on LPS induced MMP-2 activation. In addition, heat treatment of LPS had no stimulatory effect on MMP-2 activation, as shown in **Figure 4.6**. These results indicated that *P. gingivalis* LPS could directly activate MMP-2 and this effect occurred by serine protease activity of *P. gingivalis* LPS itself.

4.4 Discussion

Porphyromonas gingivalis (P. gingivalis) is one of the suspected periodontopathic bacteria and is frequently isolated from the periodontal pockets of patients with chronic periodontal disease. The lipopolysaccharide (LPS) of *P. gingivalis* is a key factor in the development of periodontitis. This study used the rapid method for isolation of the LPS. This technique is simple, requires a few of chemical reagents and instruments. The working time for LPS isolation is lesser than 6 hour whereas classical method required more than 10 days (Whesphal and Jann, 1965). The yield of LPS-isolated from the rapid method was 2 mg/ml. The isolated LPS was determined the endotoxin activity of both reference (W50) and clinical isolated strains. All isolated LPS was revealed the endotoxin unit at 4 EU/ml as judged by using E-TOXATE kit. Base on the principle of gel hardening in the reaction mixture between the lysate of the circulating amebocyte and endotoxin. This test is easy and useful technique. Because it is not only sensitive to a minute quantity of LPS, but it is also simple, rapid and less expensive to perform. Next, the isolated LPS was subjected to SDS-PAGE and visualized by silver stain to further confirm the ladder pattern of LPS from the rapid method. All strains showed ladder pattern as like as other gram-negative bacteria, which have LPS as a major component of bacterial outer membrane.

The results in this study revealed that *P. gingivalis* LPS from both reference and clinical isolated strains at the concentration of 2-20 μg/ml could induce MMP-2 activation in HPDL and HGF cells in dose-dependent manner. In the presence of NF-

kB inhibitor, LPS induced MMP-2 activation was partially inhibit. In addition, *P. gingivalis* LPS could directly activated MMP-2 to be fully active form (62 kDa). This process was inhibited by aprotinin, which is serine protease inhibitor, but not phenanthroline or EDTA. The LPS induced MMP-2 activation was occurred in 2 different way, NF-kB dependent pathway and direct activation by serine protease activity of *P. gingivalis* LPS itself. However these results were different from the previous report that demonstrated the MMP-2 activation of human uterine vascular endothelial cell (HUVEC) by LPS was NF-kB dependent process and the activation could not observed without cellular participation (Kim and Koh, 2000). The results of this study was corresponded to the recent report (Takeda et al., 2000), which demonstrated the direct activation of MMP-2 by LPS induced MMP-2 activation. After incubating the purified human proMMP-2 with *E. coli* LPS at 37 °C for 24 hours, it was found that the purified proMMP-2 (72 kDa) was processed to fully active form of MMP-2 (62 kDa). The direct activation of LPS induced MMP-2 activation was inhibited by PMSF and heat treatment of *E. coli* LPS. A number of studies investigated the effect of secreting virulence factors of bacteria on expression and MMP-2 activation. It has been shown that LPS of *T. denticola* could induced MMP-2 activation and up-regulated MT1- MMP, but not MMP-2 (Choi et al., 2003). The sonicated extracted of *P. gingivalis* could induce MMP-2 secretion and inhibit TIMP-2 production (Nakata et al., 2000). Although this study could not observed the alteration of MMP-2 expression in HGF and HPDL cells in response to *P. gingivalis* supernatant, but the similarly result that we shared is the bacterial products induced MMP-2 activation. The different results of MMP-2 expression may results from the different cell types and the different of biological active molecule (s) from different bacterial species. Another finding, bacterial cell-surface associated component from

Treponema lecthinolyticum (T. lecthinolyticum) could induced not only MMP-2 production but also MMP-2 activation in HGF and HPDL cells (Choi et al, 2001). In addition, it has been reported that lipopolysaccharide of oral spirochetes, *Treponema denticola (T. denticola),* could induce osteoclastogenesis and matrix metalloproteinase expression (Choi et al., 2003). These processes strongly suggested that the LPS are a potent stimulating factor in periodontal tissue destruction. It is noteworthy to concern about other involving effects of the lipopolysaccharide of *P. gingivalis,* which is predominant in periodontal pockets in periodontitis lesion. Since during bacterial growth and/or lysis they release small vesicle to surrounding environment. LPS was distributed to adjacent tissue and transferred to other tissue along with body fluid. Consequently results possibly lead to inflammatory reaction by activated monocytes and macrophages to secrete inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) (Gutsmann et al. 2001). The LPS of *P. gingivalis* is suspected as a key factors in the development of periodontal tissue destruction.

Although, it has been proposed that MMP-2 activation is unlike other MMPs. MMP-2 activation required MT-MMP functions and TIMP-2 molecules as trimolecular complex (Strongin et al., 1995). MMP-2 activation take place at cell surface activation and MT-MMP dependent process. The MMP-2 activation process could be inhibited by MMPs inhibitors such as EDTA, phenanthroline (Azzam and Thompson, 1992). Interestingly, the mechanism of LPS-induced MMP-2 activation requires serine protease activity of LPS itself. Since these process could inhibit by serine protease inhibitor, aprotinin and PMSF and also heat-treatment LPS. Moreover, *P. gingivalis* LPS could directly activate MMP-2 via NF-kB-independent pathway.

These results indicated that MMP-2 activation in the presence of *P. gingivalis* LPS was possibly MT-MMP independent pathway.

These data indicated that *P. gingivalis* LPS has serine protease activity, which could process the latent MMP-2 to be a fully active form of MMP-2. The evidence of these in vitro studies strongly suggested that bacterial LPS may direct and indirect activated host enzymes and consequently induced connective tissue destruction in periodontal disease. The bacterial LPS was speculated as the potent stimulator of prostaglandin releasing from macrophage in periodontal destruction (Offenbacher and Salvi , 1999). Since, the production of excessive host collagenase resulting in loss of gingival and periodontal ligament collagen and adjacent alveolar bone. It has been reported that intragingival LPS injection induces a model of periodontal disease characterized by rapid bone loss with biochemical features similar to that of naturally occurring adult periodontitis and the alveolar bone loss was inhibited by matrix metalloproteinase inhibitors in experimental periodontal disease (Ramamurthy et al., 2002). In addition the presence of LPS caused an impairment of host defense mechanism by induced necrosis of neutrophils in the gingival crevice, periodontal pocket, and oral cavity (Crawford et al., 2000). The mechanism of LPS-induced host inflammatory response associated with LBP, CD14, and TLRs. (Hayashi et al., 1999; Masaga et al., 1999; Schroder et al., 2000; Wang et al., 2000). Fibroblast is not only the major population in connective tissue but also the prime cells which responsible for inflammatory host-response such as cytokines releasing activity (Okada and Murakami, 1998). Bacteria and bacterial products including LPS act as toxifying agents and also triggering molecules. These effects may alter cell activities. The recent study was demonstrated that gingival fibroblasts from patients with chronic

adult periodontitis produced cytokines in response to changing levels of bacterial lipopolysaccharide (LPS) involving calcium dependent enzymes. The LPS also induced changing in the amount of the Ca^{++}-pump protein in gingival fibroblasts, which could alter Ca^{++}-dependent activities in these cells (Stein et al., 1999).

The rapid method of LPS isolation is beginning with lysis of bacteria by alkaline solution, and followed by digested non-specific proteins with proteinase K. Base on the principle of partition coefficient of the different molecules, two phase of phenol solution and water were mixed in hot water bath. The partition coefficient in biphasic phenol-water mixtures very often allows an almost complete extraction of proteins from aqueous solutions under controlled condition of pH and ionic strength in a one-step operation. In contrast, polysaccharide, LPS, and nucleic acids are usually water-soluble but phenol-insoluble. Therefore LPS can be precipitated from aqueous solution. LPS is one of the most putative virulence factors of *P. gingivalis*, which play a critical role in periodontal tissue destruction. The *P. gingivalis* LPS not only directly degrade host proteins but it also activates host enzyme, especially MMPs family. There are many reasons to focus on MMP-2 activation in periodontal disease. Firstly, MMP-2 was synthesized and secreted by fibroblast, which is the major population in connective tissues. Secondly, MMP-2 has a wide range of substrate specificity. Recent studies has been shown enzymatic capability of MMP-2 which including of type I collagen, type IV collagen and also denatured collagen. These data demonstrated the powerful of MMP-2 function in connective tissue destruction. Thirdly, these results also verified the LPS-induced MMP-2 activation both NF-kB dependent pathway and NF-kB independent pathway (direct activation).

Conclusion

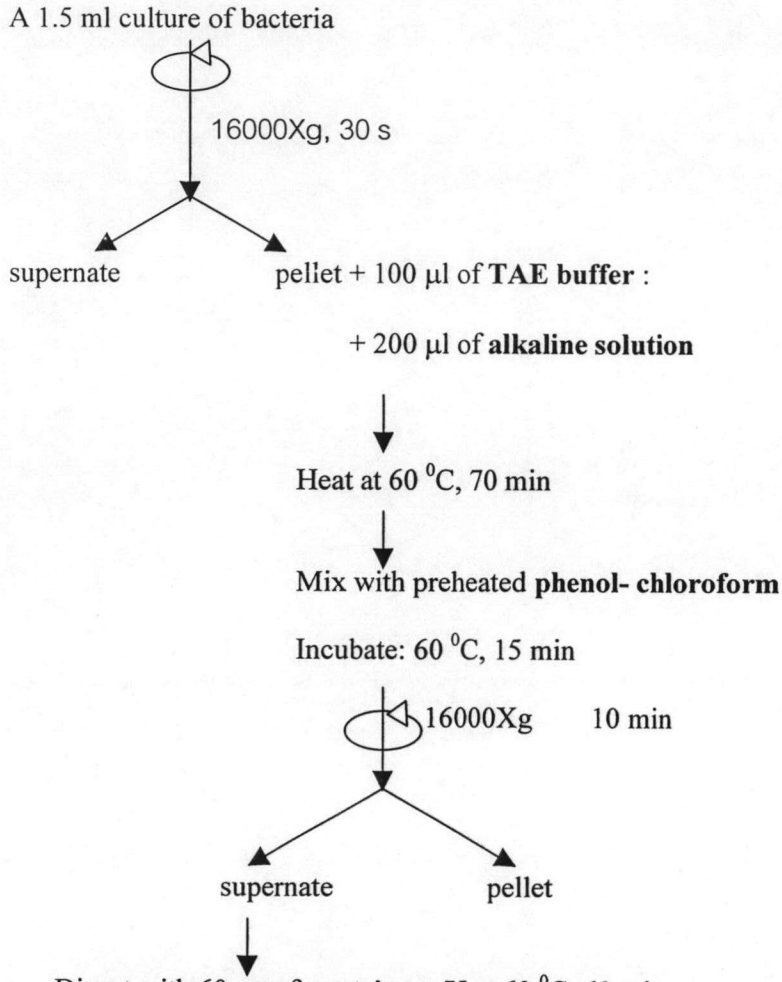
In conclusion, our results suggest that

1. Rapid method of LPS isolation is simple and practical for LPS extraction
2. *P. gingivalis* LPS-induced MMP-2 activation is occurred via NF-kB-dependent pathway and NF-kB-independent pathway.
3. Mechanism of LPS-induced MMP-2 activation required serine protease activity of *P. gingivalis* LPS because this process could be inhibited in the presence of serine protease inhibitor.

For the future study, the diversity and also the complexity of LPS structures of gram-negative bacteria were need to be clarified, particularly the serine protease activity of LPS itself.

Figure 1. Isolation of *P. gingivalis* lipopolysaccharide (LPS)
Briefly, rapid method for lipopolysaccharide isolation was suggested in previous study(Al-Hendy et al., 1991). This method required working time less than 6 hours.

Figure 4.1 Isolation of bacterial lipopolysaccharide:



- Digest with 60 µg of **proteinase K** at 60 °C, 60 min
- Mix with 200 µl H_2O + 50 µl of **3 M sodium acetate (pH 5.2)**
- Add 2 volumes of **ethanol**

- Dissolve LPS pellet in 200 µl of **50mM Tris-HCl, pH 8.0-100 mM Sodium acetate**
- Re-precipitate with 2 volumes of **ethanol**

- Dissolve LPS in 50 µl **H_2O**

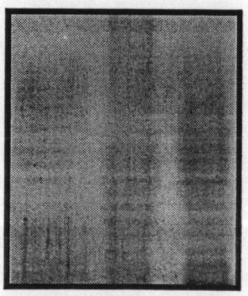
Table 4.1 Endotoxin activity measure by E-TOXATE
Extracted *P.gingivalis* LPS was subjected to determine the endotoxin activity by using the *Limulus* amebocyte lysate (LAL) test. The endotoxin activity was obtained by comparison to the endotoxin activity of standard LPS (*E. coli* LPS)

Figure 4.2 Silver staining of *P. gingivalis* LPS
Extracted endotoxin was subjected to electrophoresis at 20 mA/ slab gel until LPS in the gel were then visualized by the silver staining. All strains showed ladder pattern as like as other gram-negative bacteria, which have LPS as a major component of bacterial outer membrane.
(LPS: lipopolysaccharide ***Pg* W50:** *P. gingivalis* W50, ***Pg-1*** and ***Pg-3:*** clinical isolated *P. gingivalis* strain 1 and 3)

Table 4.1

Tube		Endotoxin in samples	Gel hardening
A	Test for endotoxin in sample		
	A1: *P. gingivalis* (W50)	2 mg/ml	+
		1 mg/ml	+
		0.5 mg/ml	-
	A2: Clinical isolated *P. gingivalis* (pt-1)	2 mg/ml	+
		1 mg/ml	+
		0.5 mg/ml	+
	A3: Clinical isolated *P. gingivalis* (pt-3)	2 mg/ml	+
		1 mg/ml	+
		0.5 mg/ml	+
B	Test for E-TOXATE inhibitor	4 EU/ml LPS: *E. coli* 0.55: B5	+
C	Negative control		-
D	Dilution of standard LPS: *E. coli*		
	D1	0.5 EU/ml	+
	D2	0.25 EU/ml	+
	D3	0.125 EU/ml	+
	D4	0.06 EU/ml	-
	D5	0.03 EU/ml	-
	D6	0.015 EU/ml	-

Figure 4.2 Silver stain of *P. gingivalis* LPS



LPS: *Pg*-W50 *Pg*-1 *Pg*-3

Figure 4.3 Effect of *P. gingivalis* LPS

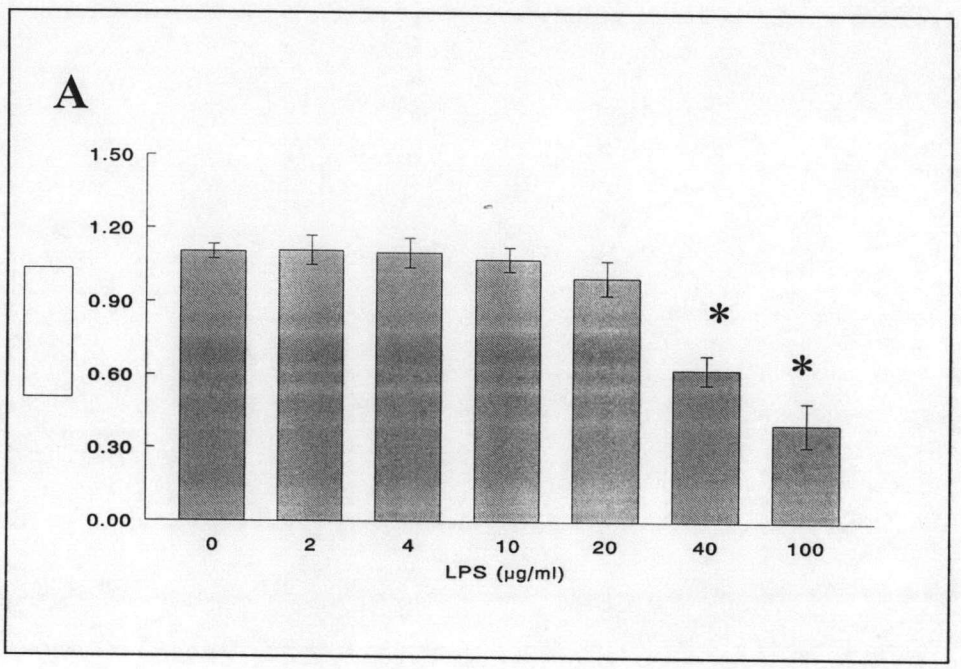
A. Cytotoxicity of *P. gingivalis* LPS

Toxicity of *P. gingivalis* supernatant determined by MTT assay cell were treated with 0, 2, 4, 10, 20, 40, 100 μg/ ml of supernatant for 24 hours. Data were shown in mean ± SD from three dependent experiments. Toxicity of LPS was observed at 20 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.(*).

B. Dose response relationship

MMP-2 activation in HPDL cells were response in dose dependent relationship. Human periodontal ligament cells were grown in the absence or presence of various concentration of *P. gingivalis* LPS (0, 2, 4, 10, 20 μg/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 4.3 Dose relationship of *P. gingivalis* LPS and MMP-2 activation



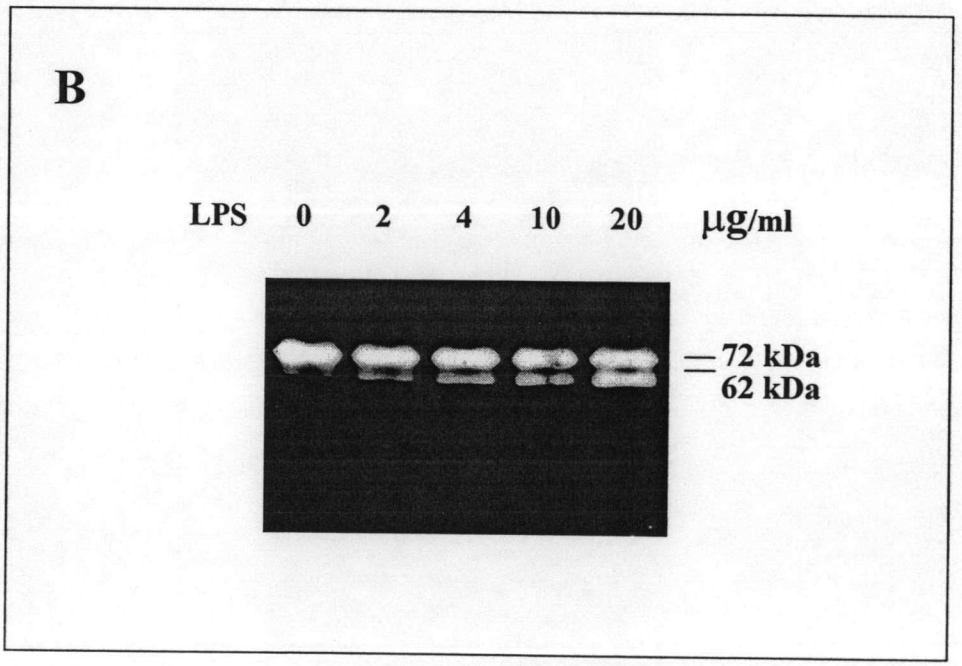


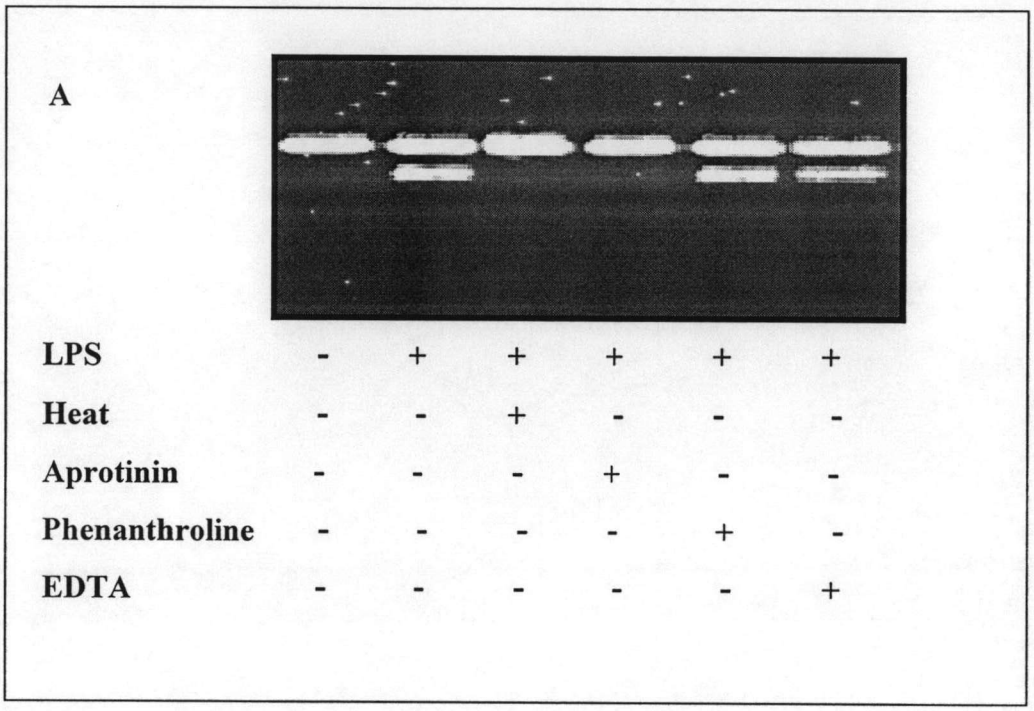
Figure 4.4 Effect of protease inhibitors on MMP-2 activation by *P. gingivalis* LPS

In the inhibitory experiments, cells were treated with proteinase inhibitors for 30 minutes before *P. gingivalis* LPS was added. Proteinase inhibitors used in the experiment included **metal chelating agents**; 10 μM phenanthroline, 2 mM Ethylene diamine tetra-acetic acid (EDTA), **serine proteinase inhibitors**; 55 ng/ml aprotinin. Another experiment cell were treated with heat treatment LPS.

A. Gelatin zymography
Conditioned medium of each was collected and determined MMP-2 activation by gelatin zymography.

B. Cytotoxicity of proteinase inhibitor
Toxicity of *P. gingivalis* LPS in the presence of each proteinase inhibitors for 24 hours. Data were shown in mean ± SD from three independent experiments. Toxicity was not observed at all 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.(*).

Figure 4.4 Effect of protease inhibitors on MMP-2 activation by *P. gingivalis* LPS



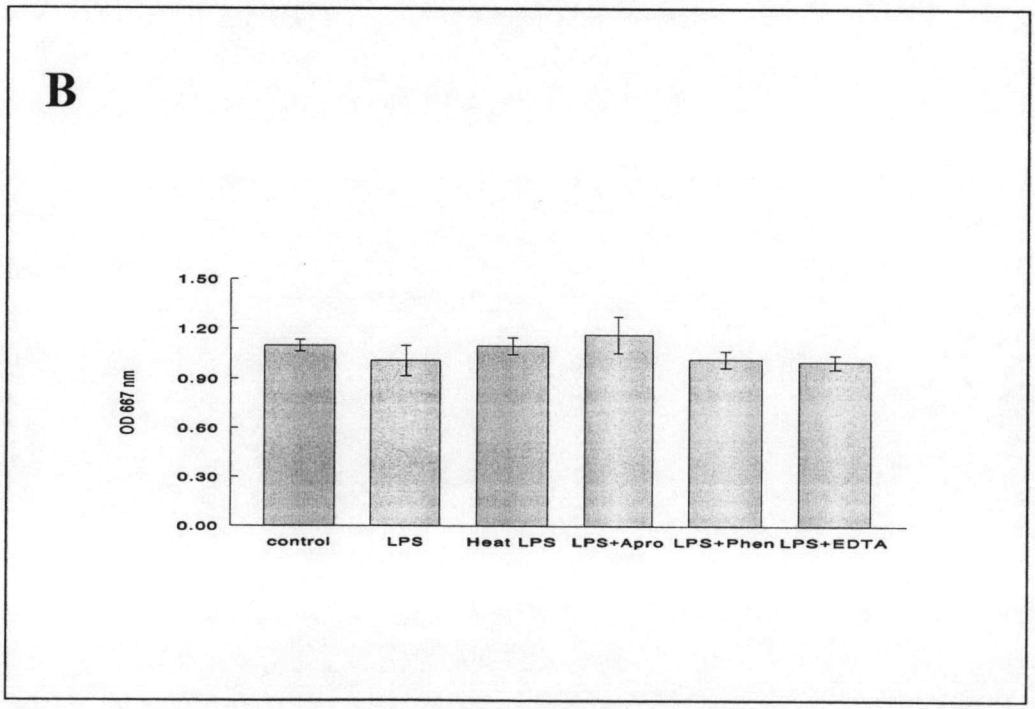


Figure 4.4 Effect of NF-kB inhibitor on MMP-2 activation by *P. gingivalis* LPS

In the next inhibitory experiments, cells were preincubated with 50 μg/ml NF-kB inhibitors (APDC) for 30 minutes before adding *P. gingivalis* LPS. **LPS** represent *P. gingivalis* LPS, **APDC**: NF-kB inhibitor, **C**: control, ***Pg* W50**: reference strain, ***Pg*-1**: *P.gingivalis* clinical isolated strain-1.

Figure 4.5 Effect of NF-kB inhibitor of MMP-2 activation by *P. gingivalis* LPS

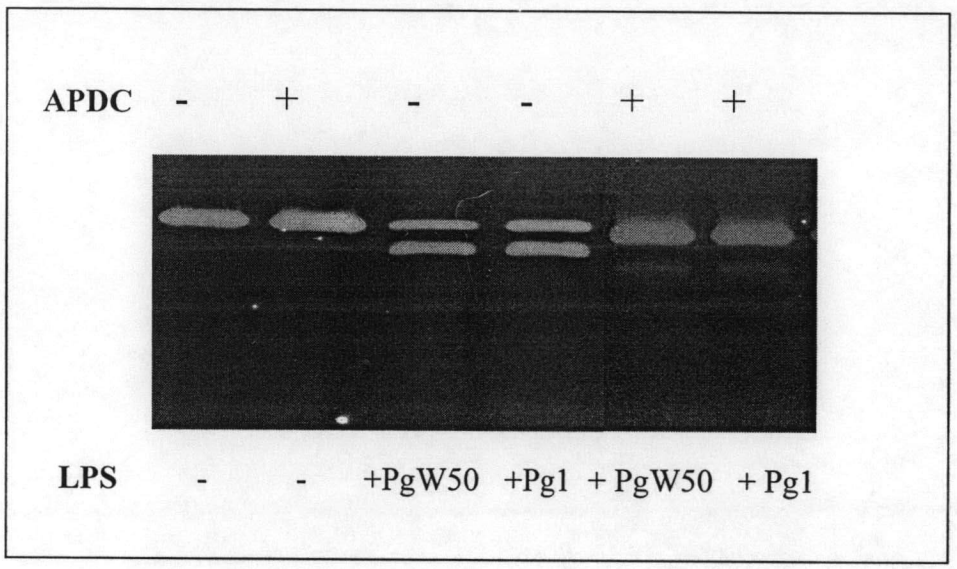
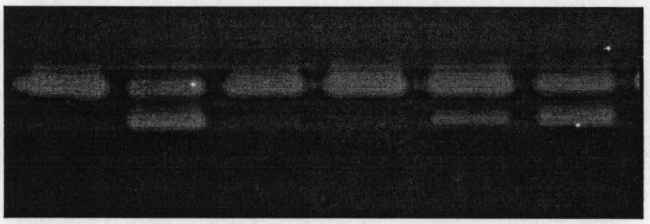


Figure 4.6 Direct activation of MMP-2 by *P. gingivalis* LPS

Cells were excluded and conditioned medium of cultured cell was incubated with *P. gingivalis* LPS in the presence or absence of protease inhibitors including **metal chelating agents**; 10 μM phenanthroline, 2 mM Ethylene diamine tetra-acetic acid (EDTA), **serine proteinase inhibitors**; 55 ng/ml aprotinin and 0.2 mM PMSF. Another experiment cell were incubated with heat treatment LPS.

Figure 4.6 Direct activation of MMP-2 by *P. gingivalis* LPS (without cell)



LPS	-	+	+	+	+	+
Heat	-	-	+	-	-	-
Aprotinin	-	-	-	+	-	-
PMSF	-	-	-	-	+	-
Phenanthroline	-	-	-	-	-	+