ผลของสารหลั่งจาก *PORPHYROMONAS GINGIVALIS*
และ *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ต่อกลไกการกระตุ้นการทำงาน
ของเอนไซม์เมทริกเมทัลโลโปติเนส-2 (เอ็มเอ็มพี-ทู) ในเซลล์เพาะเลี้ยงที่ได้จากเนื้อเยื่อปริทันต์

นางสาวสิริลักษณ์ ตีรณธนากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-17-6415-4

EFFECT OF *PORPHYROMONAS GINGIVALIS* AND *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ON THE MECHANISM OF MATRIX METALLOPROTEINASE-2 ACTIVATION IN HUMAN PERIODONTAL LIGAMENT CELLS

Miss Siriluck Tiranathanagul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Oral Biology
Faculty of Dentistry
Chulalongkorn University
Academic Year 2004

ISBN 974-17-6415-4

Thesis Title	EFFECT OF *PORPHYROMONAS GINGIVALIS* AND *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ON THE MECHANISM OF MATRIX METALLOPROTEINASE-2 ACTIVATION IN HUMAN PERIODONTAL LIGAMENT CELLS
By	Siriluck Tiranathanagul
Filed of study	Oral biology
Thesis Advisor	Associate Professor Prasit Pavasant, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctor's Degree

…….. Dean of the faculty of Dentistry
(Assistant Professor Thitima Pusiri)

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…….. Chairman
(Assistant Professor Mano Kuratana, Ph.D.)

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(Assistant Professor Rangsini Mahanonda, Ph.D.)

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(Kanokwan Nisapakultorn, Ph.D.)

…….. Member
(Nirada Dhanesuan, Ph.D.)

สิริลักษณ์ ตีรณธนากุล : ผลของสารหลั่งจาก *PORPHYROMONAS GINGIVALIS* และ *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ต่อกลไกการกระตุ้นการทำงานของเอนไซม์เมทริกเมทัลโลโปรติเนส-2 (เอ็มเอ็มพี-ทู) ในเซลล์เพาะเลี้ยงที่ได้จากเนื้อเยื่อปริทันต์. (EFFECT OF *PORPHYROMONAS GINGIVALIS* AND *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ON THE MECHANISM OF MATRIX METALLOPROTEINASE-2 ACTIVATION IN HUMAN PERIODONTAL LIGAMENT CELLS) อ. ที่ปรึกษา: รศ. ทพ. ดร. ประสิทธิ์ ภวสันต์, 128 หน้า. ISBN 974-17-6415-4

โรคปริทันต์เป็นโรคที่เกิดจากการติดเชื้อของแบคทีเรียชนิดกรัมลบ โดยมีหลักฐานพบว่าเชื้อ *P. gingivalis* และ *A. actinomycetemcomitans* เป็นเชื้อที่เป็นสาเหตุของการเกิดโรคปริทันต์ และผลของการตอบสนองของเซลล์ของร่างกายต่อเชื้อ และผลิตภัณฑ์ของเชื้อทั้งสองเป็นสาเหตุหลักของการทำลายของอวัยวะปริทันต์ การศึกษาครั้งนี้มีวัตถุประสงค์ที่จะศึกษาผลของผลิตภัณฑ์ของเชื้อทั้งสองต่อเซลล์ที่เพาะเลี้ยงจากเนื้อเยื่อปริทันต์ โดยแบ่งการศึกษาออกเป็นสองส่วน ส่วนที่หนึ่งศึกษาผลของสารหลั่งจากเชื้อ *A. actinomycetemcomitans* และ *P. gingivalis* ต่อการกระตุ้นการทำงานของเอนไซม์เอ็มเอ็มพี-ทู รวมทั้งกลไกการกระตุ้นการทำงานที่เกิดขึ้น การศึกษาทำโดยการเลี้ยงเซลล์ที่ได้จากเนื้อเยื่อปริทันต์ในสภาวะที่มีและไม่มีสารหลั่งจากเชื้อ *A. actinomycetemcomitans* และ *P. gingivalis* เป็นเวลา 48 ชั่วโมง ทำการวิเคราะห์การแสดงออกของยีนในระดับการสังเคราะห์อาร์เอ็นเอนำรหัสด้วยเทคนิค RT-PCR วิเคราะห์ระดับโปรตีนด้วยวิธีเวสเทิร์น (Western analysis) และศึกษาผลการกระตุ้นการทำงานของเอนไซม์ เอ็มเอ็มพี-ทู ด้วยวิธีไซโมกราฟี (Zymography) ผลการศึกษาในส่วนที่หนึ่งพบว่าสารหลั่งจากเชื้อ *A. actinomycetemcomitans* และ *P. gingivalis* สามารถเหนี่ยวนำให้เกิดการกระตุ้นการทำงานของเอนไซม์เอ็มเอ็มพี-ทู กลไกการกระตุ้นนี้สามารถยับยั้งได้ด้วยสารยับยั้งการทำงานของเอนไซม์เอ็มเอ็มพี ในขณะที่ตัวยับยั้งการทำงานเอนไซม์ในกลุ่มซีรีนโปรตีเอส ไม่มีผลยับยั้งการกระตุ้นดังกล่าว นอกจากนี้ยังพบว่าระดับโปรตีนของ TIMP-2 ลดลงในกลุ่มเซลล์ที่ถูกกระตุ้นด้วยสารหลั่งจาก *A. actinomycetemcomitans* ในขณะที่ระดับอาร์เอ็นเอนำรหัส และโปรตีนของ MT1-MMP เพิ่มขึ้นในกลุ่มเซลล์ที่ถูกกระตุ้นด้วยสารหลั่งจาก *P. gingivalis*

การศึกษาในส่วนที่สองเป็นการศึกษาผลของไลโปโพลีแซคคาไรด์ (แอลพีเอส) จากเชื้อ *A. actinomycetemcomitans* ต่อเซลล์ที่เพาะเลี้ยงจากเนื้อเยื่อปริทันต์ ในเวลา 36 ชั่วโมง ต่อการกระตุ้นการทำงานของเอนไซม์เอ็มเอ็มพี-ทู และการเปลี่ยนแปลงระดับของอาร์เอ็นเอนำรหัสและระดับของโปรตีนของ RANKL และ OPG ผลการทดลองพบว่าแอลพีเอสที่สกัดจาก *A. actinomycetemcomitans* สามารถกระตุ้นการทำงานของเอนไซม์เอ็มเอ็มพี-ทู โดยการกระตุ้นนี้สามารถยับยั้งโดยสมบูรณ์ได้ด้วยตัวยับยั้งการทำงานเอนไซม์ในกลุ่มซีรีนโปรตีเอส และยับยั้งบางส่วนด้วยสารยับยั้งการทำงานของนิวเคลียร์แฟคเตอร์-แคบปาบี (NF-kB) แต่ไม่สามารถยับยั้งด้วยสารยับยั้งการทำงานของเอนไซม์เอ็มเอ็มพี นอกจากนี้ แอลพีเอสจากเชื้อ *A. actinomycetemcomitans* ยังสามารถกระตุ้นการแสดงออกของยีน และเพิ่มระดับโปรตีน RANKL ในขณะที่ไม่มีผลต่อ OPG และผลการกระตุ้นนี้ถูกยับยั้งได้โดยตัวยับยั้งการทำงานเอนไซม์ในกลุ่มซีรีนโปรตีเอส และสารยับยั้งการทำงานของเอนไซม์ไซโครออกซีจิเนส

ผลของการศึกษาครั้งนี้แสดงให้เห็นถึงบทบาทที่สำคัญของเชื้อ *A. actinomycetemcomitans* และ *P. gingivalis* ต่อการทำลายเนื้อเยื่อปริทันต์ โดยสามารถเหนี่ยวนำการกระตุ้นการทำงานของเอนไซม์ เอ็มเอ็มพี-ทู ที่สร้างและหลั่งจากเซลล์เพาะเลี้ยงจากเนื้อเยื่อปริทันต์ นอกจากนี้แอลพีเอสของ *A. actinomycetemcomitans* ยังสามารถกระตุ้นการแสดงออกของ RANKL นำไปสู่การทำลายเนื้อเยื่อปริทันต์ และกระดูกรองรับรากฟันในโรคปริทันต์

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต.....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....

4276124032: MAJOR ORAL BIOLOGY
KEY WORDS: MATRIX METALLOPROTEINASE-2 ACTIVATION / *PORPHYROMONAS GINGIVALIS*/ *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* / HUMAN PERIODONTAL LIGAMENT CELLS

SIRILUCK TIRANATHANAGUL: EFFECT OF *PORPHYROMONAS GINGIVALIS* AND *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ON THE MECHANISM OF MATRIX METALLOPROTEINASE-2 ACTIVATION IN HUMAN PERIODONTAL LIGAMENT CELLS. THESIS ADVISOR: ASSOC.PROF. PRASIT PAVASANT, Ph.D., 128 pp. ISBN 974-17-6415-4

Periodontitis is initiated by specific groups of Gram-negative bacteria. Among them, *P. gingivalis* and *A. actinomycetemcomitans* have been strongly supported as etiological agents to induce host responses leading to destruction of periodontium. Therefore, the present study attempted to investigate the role of *P. gingivalis and A. actinomycetemcomitans* product on MMP-2 activation and RANKL-OPG expression in human periodontal ligament (HPDL) cells. The first part of the study was to determine the effect of *P. gingivalis* and *A. actinomycetemcomitans* supernatant on MMP-2 activation and its mechanism. Gelatin zymography, RT-PCR and Western analysis were used to detect the activation of MMP-2, expression of MT1-MMP and TIMP-2 mRNA and protein, respectively. The results showed that *P. gingivalis* and *A. actinomycetemcomitans* supernatant could activate MMP-2 in HPDL cells. Phenanthroline, an MMP inhibitor, could block the effect of supernatant from both bacteria on MMP-2 activation. In addition, *A. actinomycetemcomitans* supernatant induced the decreased amount of TIMP-2 protein while *P. gingivalis* supernatant up-regulated MT1-MMP both at mRNA and protein level. These results suggested that the MMP-2 activation by *P. gingivalis* and *A. actinomycetemcomitans* supernatant occurred via MMP-dependent pathway.

The second part focuses on the effect of *A. actinomycetemcomitans* lipopolysaccharide (LPS) on both MMP-2 activation and the expression of RANKL and OPG. The results revealed that *A. actinomycetemcomitans* LPS could activate MMP-2 but in a different mechanism from the effect of *A. actinomycetemcomitans* supernatant, since Phenanthroline could not abolish this effect. In addition, serine protease did completely. *A. actinomycetemcomitans* LPS also up-regulated RANKL expression both at mRNA and protein levels while OPG mRNA level did not change. Additionally, indomethacin and serine protease inhibitor could abrogate this effect. These results suggested the activity of serine protease of *A. actinomycetemcomitans* LPS induced MMP-2 activation and up-regulated RANKL expression.

In conclusion, the present study demonstrated the roles of *P. gingivalis* and *A. actinomycetemcomitans* virulences on periodontal tissue destruction represented here by MMP-2 activation and RANKL up-regulation.

Field of study	Oral Biology	Student's signature.....
Academic year	2004	Advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons and organizations:

My advisor, Associate Professor Dr. Prasit Pavasant, for his great guidance, constant support and encouragement throughout the course of my Doctoral degree program. To me, he has been a model supervisor and wonderful researcher.

Associate Professor Tussanee Yongchaitrakul, for her helpful advice, kindly support and generosity.

Associate Professor Dr. Erik W. Thompson, Head of invasive and metastasis unit, St. Vincent's Institute of Medical Research, Melbourne, Australia, for his kindness, valuable guidance and support, in particular, during ten months in his lab.

My thesis committee: Dr. Mano Kuratana, Dr. Rangsini Mahanonda, Dr. Kanokwan Nisapakultorn , Dr. Nirada Dhanesuan, for their valuable comment.

Thailand Research Fund through the Royal Jubilee Doctorate Program (Grant No. PHD/0024/2546), Ministry of the University Affairs of Thailand and Graduate School, Chulalongkorn University, for their financial support.

Lastly, I am sincerely grateful to my family and friends for their constant support and encouragement during this course.

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ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
AMV	Avian myeloblastosis virus
B. forsythus	*Bacteroides forsythus*
BCA	bicinchoninic acid
BHI	brain heart infusion
EMMPRIN	extracellular matrix metalloproteinase inducer
COX-2	cyclooxygenase enzyme-2
DMEM	Dulbecco's Modified Eagle medium
DNA	deoxyribonucleic acid
DTT	2,3-Dihydroxybutane-1,4-dithiol
E. coli	*Eschericia coli*
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDF	human dermal fibroblasts
HGF	human gingival fibroblasts
HPDL	human periodontal ligament cells
IL-1	interleukin-1
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MMPs	matrix metalloproteinases
NF-kB	nuclear factor-kappa B
OPG	osteoprotegerin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
PMSF	phenylmethylsulfonylfluoride
RANK	receptor activator of nuclear factor-kappa B
RANKL	receptor activator of nuclear factor-kappa B ligand
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
P. gingivalis	*Porphyromonas gingivalis*
RT-PCR	reverse transcription-polymerase chain reaction
S. salivarius	*Streptococcus salivarius*
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis

ABBREVIATIONS (Cont)

T. denticola	*Treponema denticola*
TGF-β	transforming growth factor –beta
TIMPs	tissue inhibitor of matrix metalloproteinases
TIU	trypsin inhibitory units
TNF-α	tumor necrosis factor-alpha
TSB	tryptic soy broth
SN	supernatant

CHAPTER 1

INTRODUCTION

Periodontal disease

Periodontitis is a chronic inflammatory disease that leads eventually to loss of tooth supporting structures and to loss of teeth. Considering the severity and progression of the disease there are two main categories of the disease: gingivitis and periodontitis. Clinically, gingivitis is characterized by the presence of clinical signs of inflammation, an increased of redness, swelling, a change in the position of the gingival margin and bleeding upon probing (Page, 1986) that are confined to the gingival tissue. In periodontitis lesion, beside of the sign of gingivitis, migration of the junctional epithelium down the root surface, alveolar bone resorption, and subsequent pocket formation are occurred (Page and Schroeder, 1981). Histologically, the conversion of the established lesion of gingivitis shown by the infiltration of lymphocytes in the connective tissue beneath the junctional epithelium, into periodontitis is characterized by destruction of the connective tissue attachment to the root surface and by alveolar bone loss.

The pathogenesis of these two subgroups of periodontal disease is difference. The pathogenesis of gingivitis is relatively straightforward. Bacterial accumulations initiate acute inflammatory reaction, resulting in a vascular leakage of fluid and active migration of polymorphonuclear leukocytes (PMNs, or neutrophils) out of the vessels into the tissues and into the gingival sulcus. There are also early losses of collagen just apical to the junctional epithelium following with the accumulation of lymphocyte adjacent to the junctional epithelium, and the morphological change of the fibroblasts. To this point, contributed with multifactor the tissue destruction might

be confined as a stable lesion of chronic gingivitis or progressed to be a periodontitis. The exact mechanism(s) for the transition from gingivitis to periodontitis remains unclear.

The pathogenesis of periodontitis is complicated. Numerous of details still need to be clarified. Evidences strongly supported that periodontal pathogens initiate periodontitis and play roles in disease progression both direct and indirect ways. These bacteria are capable of producing virulence substances directly damage periodontal tissue as well as triggering a number of host responses resulting in connective tissue breakdown and alveolar bone resorption. (reviewed in Nishihara and Koseki, 2004)

Periodontal pathogens

Numerous bacteria species reside in oral cavity of healthy person. The major group is Gram-positive bacteria, such as Actinomyces and Streptococci (Listgarten, 1976; Moore and Moore, 1994). In gingivitis, these two groups of bacteria are increase as well as, significantly, *Fusobacterium nucleatum* which has been implicated in causing gingivitis (Loesche and Syed, 1978; Moore et al., 1982).

Although in periodontitis, the periodontal pocket contains a large group of bacteria with over 300 species having been isolated from different individual, only a few species have been consistently found in high numbers. The early studies, Tanner et al., (1979) suggested the four bacterial species, *Porphyromonas gingivalis (P. gingivalis), Bacteroides forsythus (B. forsythus), Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans)* and *Eikenella corrodens (E. corrodens)* were the major pathogens associated with adult periodontitis. From a large

number of subsequent studies a consensus report of the World Workshop on Clinical Periodontics in (1996) concluded that three bacterial species, *P. gingivalis, B. forsythus* and *A. actinomycetemcomitans,* were not only associated with, but were the etiological agents of periodontitis. To date, the significant of these groups of Gram-negative anaerobic bacteria, *P. gingivalis, A. actinomycetemcomitans* and *B. forsythus* on initiating of the periodontal tissue destruction has also been well documented by several studies (Haffajee and Socransky, 1994; Zambon, 1996).

Actinobacillus actinomycetemcomitans

Actinobacillus actinomycetemcomitans is a Gram-negative, non-spore forming, non-motile, capnophilic, facultative anaerobic cocobacillus that grows best in an aerobic environment enriched with CO_2 5-10%.

A. actinomycetemcomitans has been associated with various forms of periodontal disease, including gingivitis (Nakagawa et al., 1994), early-onset periodontitis (Asikainen, 1986), localized juvenile periodontitis (Slots et al., 1980; Zambon et al., 1983, 1986), adult periodontitis (Slots et al., 1990), and refractory periodontitis (Rodenburg et al., 1990). Although this microorganism may also be found in subject without evident signs of periodontal destruction, it occurs at a lower frequency in healthy subjects compared with patient with periodontitis (Muller et al., 1996).

A. actinomycetemcomitans appears to employ multiple products to inactivate or evade immune defenses. The most effective virulence factors of this microorganism is leukotoxin (Baehni et al., 1979; Lally et al., 1999; Narayanan et al., 2002) which cause the target cells to undergo apoptosis by a mechanism involving

mitochondrial perturbation (Korostoff et al., 2000a). In addition *A. actinomycetemcomitans* produces collagenase (Robertson et al., 1982; Rozanis, 1982, 1983) and epitheliotoxin (Zambon, 1985) that are capable of directly causing tissue destruction. Besides these virulence factors, *A. actinomycetemcomitans* LPS also imparts virulence capabilities to this organism (Kiley and Holt, 1980; Saglie et al., 1990).

Porphyromonas gingivalis

Porphyromonas gingivalis is an anaerobic, asaccharolytic, gram-negative, black-pigmented coccobacillus. *P. gingivalis* has been commonly isolated from periodontal disease sites (Slots and Genco, 1984; Dzink et al., 1988; Slots and Listgarten, 1988), and patients with periodontitis have elevated serum antibodies specific to this pathogen (Vincent et al., 1985; Ebersole et al., 1986; Martin et al., 1986). *P. gingivalis* exhibits a number of virulence factors, including fimbrial adhesins, lipopolysaccharide (LPS), capsule, collagenase, and cysteine proteases with trypsin-like activity to adhere and destroy host tissue. Among these factors, one distinguishing property of these organisms that has been implicated in periodontal destruction is the expression of potent protease activity. *P. gingivalis* proteases characterized as trypsin-like proteases, are classified to 3 major groups: RgpA , RgpB (both also called Arg-gingipains) and Kgp (Lys-gingipain), which are specific for arginine and lysine-containing peptide bonds, respectively. These proteases have several functions effecting host tissue. Specific examples of tissue degradation and attenuation of host defense mechanisms include the degradation of extracellular matrix proteins, activation of Matrix metalloproteinases (MMPs), inactivation of

plasma protease inhibitors, cleavage of cell surface receptors, activation or inactivation of complement factors and cytokines, and activation of the kallikrein kinin cascade (Travis et al., 1997).

Lipopolysaccharides (LPS)

One of the most detrimental virulence factors of Gram-negative bacteria is Lipopolysaccharides (LPS).

Lipopolysaccharides (LPS) or endotoxin are major constituents of the outer membrane of most Gram-negative bacteria (Rietschel and Brade, 1992; Rietschel et al., 1994). Since it consists of polysaccharides and a lipid part, therefore, it was termed lipopolysaccharides. LPS is necessary for bacterial viability (Raetz et al., 1991), as a shield from environmental conditions including cellular host defense mechanism. During Gram-negative bacterial infections, systemically, LPS is capable of inducing all the symptoms of septic shock, including fever, vascular collapse and death. Locally, lipopolysaccharide can stimulate various cell types including monocytes, macrophages, polymorphonuclear leukocytes (PMN), B-lymphocyte, vascular cells, epithelial cell and fibroblasts. These cells react to LPS in several ways such as mediator production, phagocytosis, proliferation and differentiation. For example, LPS can trigger monocytes to release inflammatory mediators namely interleukins -1, -6, -8, and tumor necrosis factor that increase local destruction of the connective tissues structure elements (Haeffner-Cavaillon et al., 1989; Laude-Sharp et al., 1990; Agarwal et al., 1995).

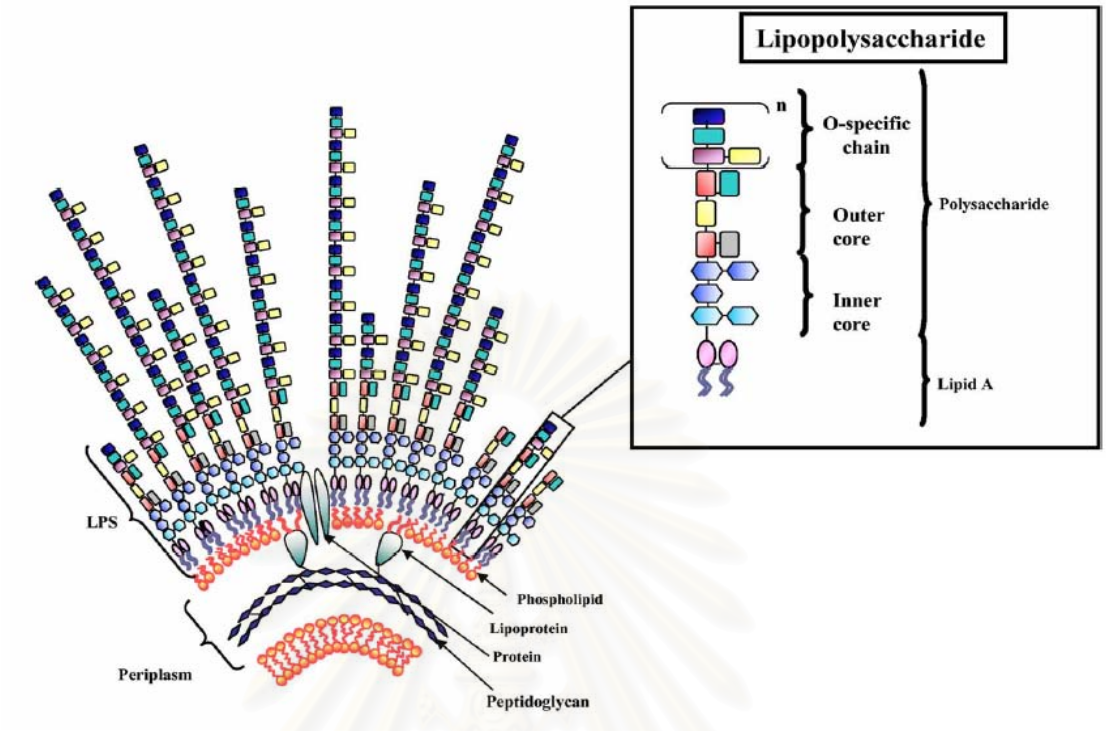


Figure 1.1 The structure of Lipopolysaccharides
(adapted from Caroff and Karibian, 2003)

Structure of bacterial lipopolysaccharides

The general structure of LPS is shown in Figure 1.1, represented by the O-specific chain, the core oligosaccharide and the lipid A.

In part of polysaccharide, LPS consists of two regions: O-specific chain and core region. O-specific chain is a heteropolysaccharide, made up by repeating units, which contains between two and eight sugar monomers. The composition and length of the polysaccharides vary among bacteria. This region is responsible for serotypic specificity of bacterial strain (Westphal et al., 1983).

Core region shows less variation in the composition than in the structures of O-specific chains. The core oligosaccharide contains the unique sugar 2-keto-3-deoxyacetonate (KDO) that links this region to lipid A.

Lipid A is a D-glucosamine-based, β(1→ 6)-linked disaccharide phospholipids (Brozek and Raetz, 1990) which anchors the molecule in the outer membrane and represent the toxic part of the molecule (Galanos et al., 1985).

Lipopolysaccharide recognition and signal transduction

There are many studies on how cells response to LPS, most of them were took on monocytes and macrophages. To date, the most well know LPS receptor is CD14.

CD14 has two forms namely membrane and soluble form. Membrane CD14 (mCD14) is a 55-kDa glycoprotein present at the cell surface, embedded in the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor. Another form of CD14, sCD14, 42-kDa single polypeptide chain (Bazil et al., 1986), is resulted either from the releasing by activated cells or enzymatically cleaved from the membrane of CD14 expressing cells (Labeta et al., 1993).

Originally CD14 was defined as a LPS receptor, up to now, various bacterial components are discovered to bind CD14, for instance, peptidoglycan (Pugin et al., 1994; Weidemann et al., 1994, 1997; Dziarski et al., 1998), Lipoteichoic acids (Cleveland et al., 1996) , Lipoarabinomannan (Zhang and Rom, 1993; Savedra et al., 1996), and Rhamnose-glucose polymers (Soell et al., 1995).

The activation of cells by LPS occurs, initially, via binding of LPS and LPS-binding protein (LBP). LBP is a 60-kDa glycoprotein produced by liver to the circulation, functioning as a lipid transfer molecule catalyzing movement of phopholipids, in particular LPS monomers from LPS aggregates to both mCD14 and sCD14 and phospholipids (especially high-density lipoproteins, HDL) (Tobias et al., 1993; Hailman et al., 1994; Wurfel et al., 1994; Yu and Wright, 1996). LBP forms a

complex together with LPS, enhances the binding to CD14 and reduces the concentration of LPS necessary for activation.

In CD14-negative cells such as endothelial and smooth muscle cells, sCD14 appears to play an important role in LPS signaling in concert with LBP to form complex and transfer to 80 kDa LPS binding protein on the cell surface (Pugin et al., 1993; Schletter et al., 1995).

Besides, other LPS receptors have been described, CD18 (Wright, 1991; Ingalls and Golenbock, 1995), the scavenger receptor (Hampton et al., 1991). Other LPS binding proteins on monocytes/macrophages are a 73 kDa protein (Lei and Morrison, 1988; Lei et al., 1991) and a 40 kDa protein (Kirikae et al., 1991). However, the certain role of these receptors on LPS signaling remains to be investigated.

In LPS induced-signal transduction via CD14, since CD14 lacks transmembrane and intracellular domains, it could not directly generate the intracellular signal. Apparently, the main function of CD14 is to catalyze the transfer of LPS from extracellular space to the membrane where it associates with a complex of receptors (Raetz et al., 1991; Lee et al., 1993), Toll-like receptor 4 (TLR4)–Myeloid differentiation protein-2 (MD-2) complex (da Silva Correia et al., 2001).

Toll-like receptors (TLRs) are cell surface receptors for specific patterns of microbial components. Ten members of the TLR family (TLR1-10) have been reported so far (Takeuchi and Akira, 2001). TLRs were shown to induce the expression of genes involved in inflammatory responses. Each TLR was found to bind to specific microbial components, for example, LPS. Although TLR4 has been shown to be responsible for LPS signal (Chow et al., 1999; Hoshino et al., 1999), TLR2 has

been also found to mediate *P. gingivalis* LPS signal (Hirschfeld et al., 2000; Bainbridge and Darveau, 2001)

Downstream signaling components of TLR4 include MyD88, IRAK and TRAF6. The signal finally activates MAP kinases and NF-kB transcription factors (Muzio et al., 1998). However TLR4 alone is not sufficient for LPS signaling. MD-2, an 18-25 kDa protein that is associated with TLR4 on the cell surface, was found to be important for LPS recognition by TLR4 (Shimazu et al., 1999; da Silva Correia et al., 2001b; Ohnishi et al., 2001; Schromm et al., 2001; Visintin et al., 2001; Nagai et al., 2002).

Other LPS-activation cluster have been identified for example on LPS-activated monocyte, an activation cluster composed of CD14, TLR4, CD55, CD16a, CD11b/CD18, Fcγ-receptors (CD32 and CD64), FcγRIIIa, CD36 and CD81 (Pfeiffer et al., 2001). Accumulating evidence supported that the responses to different pathogens vary depending on cell type, composition of supramolecular activation clusters and intracellular adaptor molecules (Triantafilou and Triantafilou, 2002).

As different receptors are recruited to the site of ligation forming an activation cluster, multiple signaling cascades are triggered. LPS has been shown to be able to activate the NF-kB, ERK1/2 and SAPK/JNK pathways (Irie et al., 2000; Lee et al., 2000).

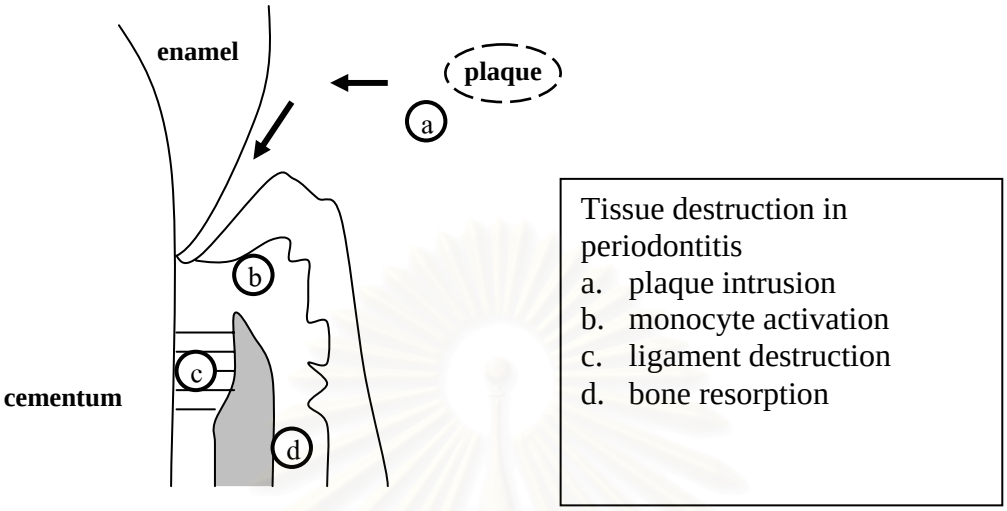


Figure 1.2 Tissue destruction in periodontitis
(adapted from Reynolds and Meikle, 1997)

Tissue destruction in periodontal disease

The periodontium is defined as those tissue supporting and investing the tooth consisting of cementum, periodontal ligament (PDL), bone lining the alveolus (socket) and the part of the gingival facing the tooth. According to the component of the periodontal tissue, the destruction can be classified into two categories, soft and hard tissue destruction. Soft tissue destruction, connective tissue breakdown, is responsible by proteolytic enzyme and hard tissue destruction, bone resorption, is responsible by the coupling function of osteoblast and osteoclast. Although the degradation mainly results from host immune and inflammatory response to the microbial challenge, indisputably, direct degradation from bacterial proteolytic enzymes or toxins still partly play roles in this mechanism.

Since subgingival Gram negative bacteria use proteins for their nutrition, they possess proteolytic enzyme to breakdown proteins to peptides and amino acids, which they can utilize. The main structural proteins of gingival connective tissue and

periodontal ligament are collagen and proteoglycan. The proteolytic enzymes that produced by periodontal pathogens include collagenase from *P. gingivalis*, *A. actinomycetemcomitans* and spirochetes (Robertson et al., 1982), elastase-like enzyme from spirochetes (Uitto et al., 1986), trypsin-like enzyme from *P. gingivalis, B. forsythus, T. denticola* and other spirochetes (Suido et al., 1986; Gazi et al., 1997), Chemotrypsin-like enzymes from *T. denticola* and Capnocytophaga species (Uitto et al., 1988; Uitto et al., 1989; Gazi et al., 1997), aminopeptidases from Capnocytophaga species (Suido et al., 1986; Gazi et al., 1997) and *T. denticola* (Gazi et al., 1997) and dipeptidyl peptidases from *P. gingivalis, P. intermedia* and Capnocytophaga species (Gazi et al., 1995, 1997). Besides bacterial enzymes, some bacteria can produce other virulence factors; for instance, leukotoxin produced by *A. actinomycetemcomitans* can damage PMNs and macrophages (Tsai et al., 1979).

Although these bacteria found in the subgingival plaque are capable of directly destroying the periodontal tissue by their proteolytic enzymes, for example collagenase, but the primary collagenase activity in the periodontium appears to be due to enzymes released from PMNs, macrophages, and fibroblasts. It is now widely accepted that the host response to these bacteria and their products entering the tissues and activating inflammatory and immune processes namely increasing the secretion of inflammatory cytokines and host proteolytic enzymes, is the major cause of the periodontal destruction (Sorsa et al., 1992; Birkedal-Hansen, 1993; DeCarlo et al., 1997).

In addition, although lipopolysaccharide from subgingival bacteria is capable of directly activating bone resorption in tissue culture systems, the same lipopolysaccharide activate many other host mechanisms that appear to be primarily

responsible for the bone destruction seen in periodontitis. However, the quantity of bacterial plaque and the types of bacteria found in the plaque do not by themselves appear to explain the severity of clinical disease. The significant factor in the disease process is the susceptibility of the host.

During disease progression, periodontal pathogens can evade the host clearance mechanisms (complement, antibodies, and neutrophils), while shedding vesicles containing microbial toxins, proteases, and lipopolysaccharide (LPS). These molecules penetrate the tissues, stimulating monocytes which secrete mediators of inflammatory, including prostagladin E2 (PGE_2), thromboxane B2 , interleukins -1, -6 and -8, tumor necrosis factor (TNF), and collagenase. These mediators of inflammation then activate vascular smooth muscle cells, fibroblasts, more monocytes, and osteoclasts to produce matrix metalloproteinases and stimulate bone resorption.

Role of MMPs in periodontal disease

Among host proteolytic enzymes found in periodontitis lesion, Matrix metalloproteinases (MMPs) are marked found and play important roles in periodontal tissue destruction. In periodontitis, several types of MMPs were detected in gingival crevicular fluid (GCF), saliva and gingival tissue. In gingival tissue of periodontitis patients, an increase of several MMPs was found including MMP-1, -2, -7, -8 -9, -13, and MMP-14 (Ingman et al., 1994; Aiba et al., 1996; Kubota et al., 1996; Tervahartiala et al., 2000; Dahan et al., 2001; Ejeil et al., 2003; Smith et al., 2004). MMP-2, -7, -8 and MMP-13 were expressed in gingival sulcular epithelium (Tervahartiala et al., 2000). MMP-9 has also been detected in junctional and pocket

gingival epithelial cells, polymorphonuclear neutrophils (PMN) and as a scattered deposit along connective tissues of periodontitis-affected gingival tissues (Smith et al., 2004).

In saliva and GCF, collagenases (MMP-1 and -8) and gelatinases (MMP-2 and -9) were marked found higher than normal (Ingman et al., 1993; Ingman et al., 1994; Ingman et al., 1996; Nomura et al., 1998). The amount of gelatinases (MMP-2 and MMP-9) was elevated during periodontal disease (Makela et al., 1994).

Besides, GCF MMP-8 was used to differentiate periodontitis from gingivitis and healthy sites as well as to monitor treatment of periodontitis (Mantyla et al., 2003)

MMP-2 in periodontal disease

An significant role of gelatinase A (MMP-2) has been pointed up since the finding that in periodontitis lesion, beside of the latent form, the increased active form of MMP-2 was detected (Korostoff et al., 2000b; Ejeil et al., 2003). Moreover, ligature-induced periodontitis in rat model (Achong et al., 2003), an increase of MMP-2 and MT1-MMP was found at day 21 after ligature placement by using gelatin zymography, Northern blot and in situ hybridization, indicating the roles of MT1-MMP in concert with MMP-2 in periodontal disease.

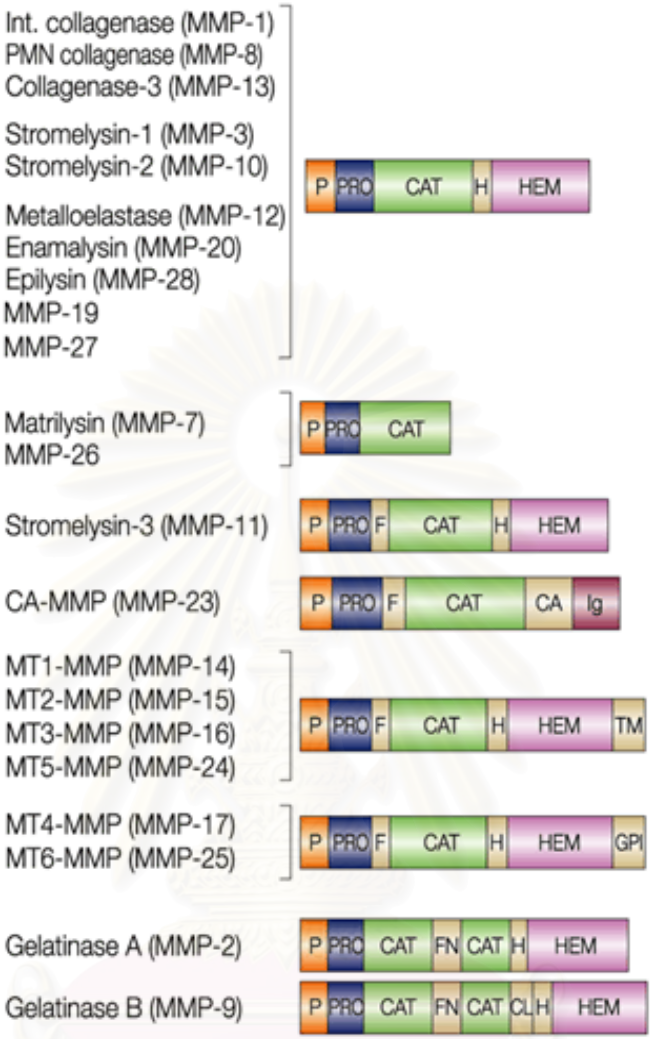
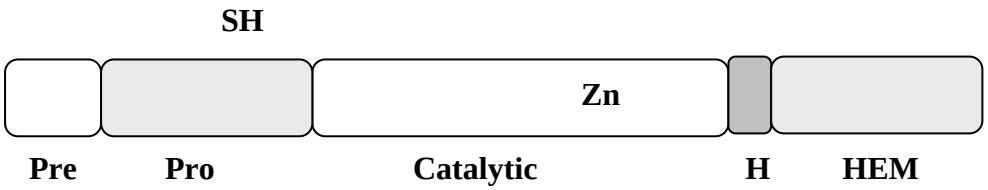


Figure 1.3 The matrix metalloproteinase (MMP) family
(adapted from Brinckerhoff and Matrisian, 2002)
CA, cysteine array; CAT, catalytic domain; CL, collagen-like domain; F, furin-cleavage consensus sequence; FN, fibronectin-like repeats; GPI, glycosyl phosphatidylinositol linkage signal; H, hinge domain; HEM, hemopexin domain; Ig, immunoglobulin-like domain; P, leader sequence; PRO, pro-domain; TM, transmembrane domain.

Table 1.1 Members of Matrix Metalloproteinase Family

MMP number	Common name	Substrate
1	Interstitial collagenase	Types I,II, III, VI, VIII and X collagen, gelatin
2	Gelatinase A	Gelatin, types I, IV, V and X collagen, laminin V
3	Stromelysin 1	Types III, IV, IX and X collagen, gelatin, proMMP-1, laminin, proteoglycans
7	Matrilysin	Gelatin, fibronectin, proMMP-1
8	Neutrophil collagenase	Types I, II, III, VII, and X collagen
9	Gelatinase B	Gelatin, types I, IV, V, and X collagen
10	Stromelysin 2	Types III, IV, IX, and X collagen, gelatin, proMMP-1, laminin, proteoglycans
11	Stromelysin 3	Alpha-1-antiprotease
12	Metalloelastase	Elastin
13	Collagenase 3	Types I, II, III, VII, and X collagen
14	MT1-MMP	ProMMP-2, gelatin, collagens, laminin-5
15	MT2-MMP	ProMMP-2, gelatin
16	MT3-MMP	ProMMP-2, collagens
17	MT4-MMP	TNF-α
18/19	Collagenase 4	?
20	Enamelysin	Amelogenin
21	XMMP (Xenopus)	?
22	CMMP (chicken)	?
23		?
24	MT5-MMP	?
25	MT6-MMP	?
26	Endometase, Matrilysin-2	?
27		?
28	Epilysin	?

(adapted from Kleiner and Stetler-Stevenson, 1999; Sternlicht and Werb, 2001)



Pre = signal sequence
Pro = propeptide with a free zinc-ligating thiol (SH)
Catalytic = Catalytic domain with zinc-binding site (Zn)
H = hinge
HEM = hemopexin-like domain

Figure 1.4 MMPs structure (adapted from Sternlicht and Werb, 2001)

Structure and functions of MMPs

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases produced in latent forms (pro-MMP) requiring activation for catalytic activity. These enzymes are capable of degrading all types of collagen, gelatin, elastin, fibronectin, laminin and proteoglycan core protein at neutral pH. Many cells are able to produce the enzyme in MMP family; for example, endothelial cells, keratinocytes, fibroblasts, osteoblasts, chondrocytes, macrophages and monocytes, and polymorphonuclear leukocytes. MMPs participate in connective tissue remodeling during normal process such as embryonic development, bone growth, and wound healing. Additionally they have been implicated in several disease processes such as inflammation and tumor progression (Woessner, 1991; Birkedal-Hansen, 1993; Ellerbroek and Stack, 1999). The MMP family (Figure 1.3 and Table 1) is composed of at least 25 enzymes separated in 4 groups: (i) the collagenases (MMP -1, 8 and 13), (ii) the gelatinases A and B (MMP-2 and 9), (iii) the stromelysin 1 and 2 (MMP-3, 7, 10 and MMP-12), (iv) the membrane-type MMPs (MT1 to MT6-MMP).

All MMPs share a basic structural organization comprising several domain motifs including predomain as a signal sequence, propeptide, catalytic, hinge, C-terminal hemopexin-like domain and, additional domain according to MMP subgroup (Figure 1.3); for example, the transmembrane domain in MT-MMPs.

The propeptide domain (about 80 amino acids) has a conserved amino acid sequence PRCG(V/N)PD. The cysteine (Cys) within this sequence termed the "cysteine switch", ligates the catalytic zinc to maintain the latency of pro-MMPs (Van Wart and Birkedal-Hansen, 1990; Birkedal-Hansen, 1993). The displacement of the cysteine residue by several means; for example, oxidation, proteolytic cleavage, mercurial and gold compound, would turn on the enzyme activity.

The catalytic domain consists of approximately 170 amino acids, containing a zinc-binding motif HEXGHXXGXXHS, which are responsible for co-ordination of the zinc atom. This domain has additional structural zinc ion and 2-3 calcium ions, which are required for the stability and the expression of enzyme activity (Nagase and Woessner, 1999).

The C-terminal hemopexin-like domain (about 210 amino acids) is essential for collagenases to cleave triple helical interstitial collagens (Bode, 1995). One of the roles of this domain is interaction with the tissue inhibitors of matrix metalloproteinase (TIMPs), a family of specific MMP inhibitor (Overall et al., 1999). In MMP-2 this domain is also required for the cell surface activation of pro-MMP-2 by MT1-MMP (Murphy et al., 1992; Strongin et al., 1995).

The function of the proline-rich hinge domain connecting the catalytic and the hemopexin domain is not known. The transmembrane domain comprising approximately 25 amino acids is found in the MT-MMPs to anchor those enzymes to

the cell surface. Due to the enormous capability of these enzymes on extracellular matrix degradation, the regulation of their expression and function are very important. Matrix metalloproteinases are regulated in several levels: transcription level, inhibition level, however, the most critical level is activation level.

At the transcription level, many cytokines and growth factors such as TNF-α , IFN-γ and IL-4 modulate the transcription of MMPs. Moreover, extracellular matrix metalloproteinase inducer (EMMPRIN) induces production of MMPs such as MMP-1, MMP-2 and MT1-MMP by stromal fibroblasts (Bordador et al., 2000; Dalberg et al., 2000; Sun and Hemler, 2001).

At the inhibition level, specific inhibitors, the tissue inhibitor of metalloproteinases (TIMPs) reduce the proteolytic activities of MMPs. Four different TIMPs have been found: TIMP-1,-2,-3 and TIMP-4. TIMPs are widely distributed in tissues and fluids and are expressed by many cell types including fibroblasts (Stricklin et al., 1983), keratinocytes (Lin et al., 1987), monocytes/macrophages (Campbell et al., 1987) and endothelial cells (Herron et al., 1986). In the inhibitory role, all TIMPs form tight 1:1 complexes with MMPs. Besides, TIMPs are involved in complicated biological functions such as induction of cell morphological changes, stimulation of growth in some cell types and inhibition of angiogenesis (Hayakawa et al., 1992; Nemeth et al., 1996; Gomez et al., 1997; Shoji et al., 1997; Fassina et al., 2000; Guedez et al., 2001). TIMP-2, a 22 kDa unglycolated protein, is expressed by several cell types; for example, fibroblasts and endothelial cells (DeClerck and Laug, 1986; Herron et al., 1986; Stetler-Stevenson et al., 1989). Besides the inhibitory function, TIMP-2 also particitpates in MMP-2 activation mechanism on the cell surface.

Finally, the most seemingly important regulation level of the matrix metalloproteinases is the activation level. Apart from a few members activated by furin, most MMPs are secreted as inactive pro-enzymes and need activation process to function. Secreted pro-MMPs are activated by proteases and in vitro by chemical agents such as SH-reactive agents, mercurial compounds, reactive oxygen, and denaturants. In all cases, activation requires the disruption of the Cys-Zn^{2+} (cysteine switch) interaction, and the removal of the propeptide. These processes are often in a stepwise manner (Nagase, 1997). The cysteine switch model proposes that the, in chemical activation, Cys-Zn^{2+} interaction at the active site transiently dissociate, thereby allowing Cys to react with SH reagents. The modification of Cys then prevents the reassociation of Cys and Zn^{2+} following by autolytically processing of the propeptide. In vivo, the activators for this mechanism are tissue or plasma proteases, such as plasmin, or opportunistic bacterial proteases. These proteases cause proteolytic activation of MMPs by removing the propeptide.

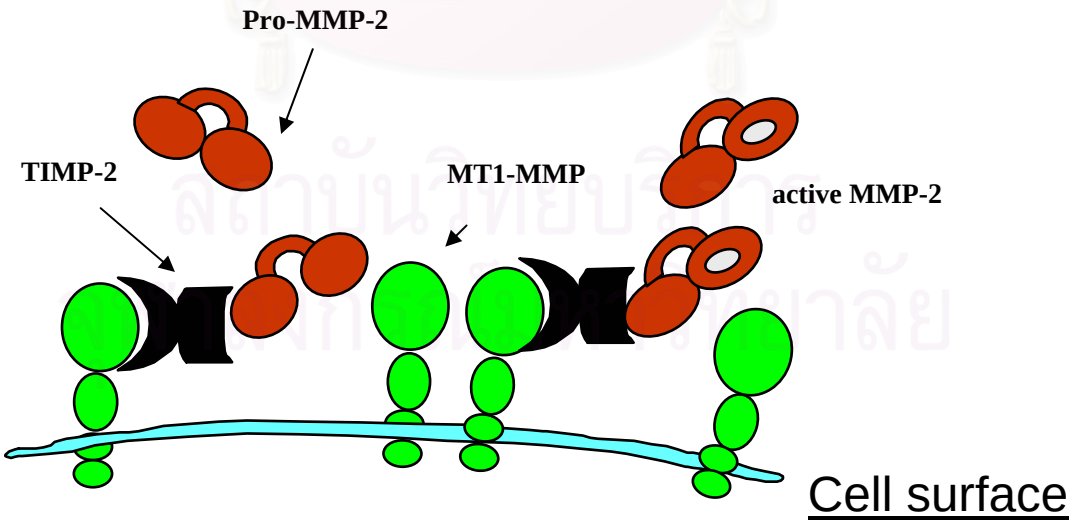


Figure 1.5 MMP-2 activation on the cell surface
(adapted from Strongin et al., 1995)

Matrix metalloproteinase-2 (MMP-2)

Matrix metalloproteinase (MMP)-2 (type IV collagenase; gelatinase A; 72kDa gelatinase) degrades denatured collagens, gelatin, as well as type IV collagen, a principle structural component of basement membrane. Moreover, MMP-2 is able to digest type I, II and III collagens (Aimes and Quigley, 1995; Patterson et al., 2001). MMP-2 is secreted mainly by fibroblasts, the majority cells of connective tissue.

Structurally, MMP-2 contains the propeptide, catalytic domain inserted with three repeats of a fibronectin-like domain and hemopexin domain. The fibronectin domain functions as a binding site for the substrate namely gelatin, collagens and laminin (Allan et al., 1995). Among all MMPs, MMP-2 has a unique activation mechanism. Activation of pro-MMP-2 has been shown to occur at the cell surface (Figure 1.5). N-terminal domain of TIMP-2 binds to MT1-MMP prior to using C-terminal domain binding to the hemopexin domain of pro-MMP-2 forming three-molecular complex. Then nearby free MT1-MMP cleaves the pro-domain of pro-MMP-2, at the Asn37-Leu38 bond generating an intermediate species, followed by an autocatalytic conversion of the intermediate into a fully active enzyme (Atkinson et al., 1995; Strongin et al., 1995; Imai et al., 1996; Sato et al., 1996; Butler et al., 1998; Kinoshita et al., 1998; Murphy et al., 1999). Pericellular TIMP-2 concentration is critical for MT1-MMP mediated activation, such that insufficient TIMP-2 results in failure to localize pro-MMP-2 to the cell surface, while excess inhibitor reduces the population of free MT1-MMP needed to activate pro-MMP-2.

Obviously, from the MMP-2 activation model, the balance of these molecules: MMP-2, TIMP-2 and MT1-MMP, is important to stipulate the level of activation and degradation ability of MMP-2. The imbalance of these molecules

especially MMP-2 and TIMP-2 is possibly involved in invasiveness of cancers such as malignant glioma (Hur et al., 2000; Nakada et al., 2001).

RANKL and OPG in periodontal disease

Bone remodeling occurs throughout life time, the balance between bone formation and bone resorption has to be precisely regulated to maintain the bone mass and support the function of bone. There are two major groups of cells responsible for these process namely osteoblasts and osteoclasts. The regulation of bone resorption involves a complicated set of hormonal and/or cytokine interactions that initially stimulate osteoblasts and stromal cells, which then elaborate factors that signal preosteoclasts to proliferate and differentiate to be mature osteoclasts and leading to degrading of the bone.

Osteoclasts are multinucleated giant cells with the capacity to resorb mineralized tissues. These osteoclasts are derived from hematopoietic precursor cells belonging to the monocyte/macrophage lineage. Osteoclast development has been reported to be regulated by several molecules such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor – kappa B ligand (RANKL), and a decoy receptor of RANKL, osteoprotegerin (OPG).

Osteoblasts are derived from undifferentiated bone marrow mesenchymal precursor cells. The major functions of the osteoblast are to produce the components of the bone matrix, largely type I collagen with lesser amounts of proteoglycans and glycoproteins, and to catalyze the calcification of the matrix. Beside of the bone formation function, these cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitors.

In bone resorption mechanism, initially osteoblasts release collagenase to remove the nonmineralized organic matrix, which covers bone surfaces. The osteoclast is then attracted to this site, seals itself onto the calcified matrix, and acidifies it by pumping protons outwards, thus solubilizing the calcium salts. The osteoclast then releases various lysosomal enzymes to remove the exposed organic matrix. The solubilization of the matrix is believed to release bound growth factors which stimulate mesenchymal cell to proliferate and differentiate into preosteoblasts and osteoblasts which can then replace the previously removed matrix.

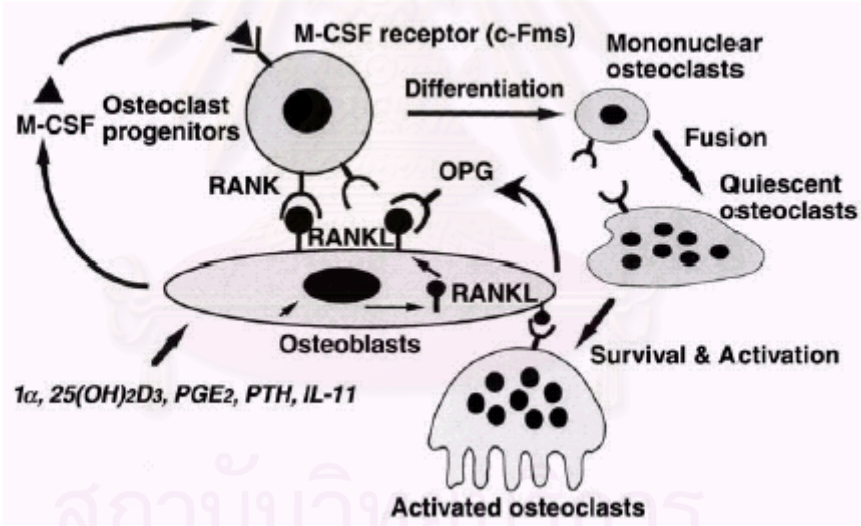


Figure 1.6 The Roles of RANKL, RANK and OPG on osteoclastogenesis (adapted from Udagawa, 2002)

Osteoclastogenesis is occurred and regulated with the association of several molecules namely RANKL, RANK, OPG and M-CSF. Receptor activator of NF-kB ligand (RANKL, also referred to as ODF-osteoclast differenting factor, OPGL-

osteoprotegerin ligand, TRANCE-TNF related activation induced cytokine, and TNFSF11-tumor necrosis factor (ligand) superfamily 11) is a membrane bound factor which is produced by osteoblasts and stromal cells in response to a variety of signals such as calcitriol (Vitamin D) (Miura et al., 2002), parathyroid hormone (PTH) (Lee and Lorenzo, 1999), TNF-α (Hofbauer et al., 1999b), glucocorticoids (Chung et al., 2001), PGE_2 (Li et al., 2002), Interleukin 1 and interleukin 11 (Hofbauer et al., 1999a; Aubin and Bonnelye, 2000), Thyroid hormone (Miura et al., 2002), Lipopolysaccharide (Kikuchi et al., 2001), bacterial CpGp-DNA and Viral double stranded DNA (Suda et al., 2002), histamine (Deyama et al., 2002), fibroblast growth factor-2 (FGF-2) (Chikazu et al., 2001), Insulin like growth factor-1 (Rubin et al., 2002), and low gravity (Kanematsu et al., 2002).

The RANKL binds to the cytoplasmic membrane receptor RANK (receptor activator of NF-kB), which is a member of the tumor necrosis factor (TNF) receptor superfamily and subsequently induces both osteoclast differentiation and activation. This event is blocked by OPG, a soluble decoy receptor for RANKL, subsequent preventing bone resorption. There are many studies revealing the critical roles of there molecule on bone resorption. Overexpression of OPG in transgenic mice results in osteopetrosis (Min et al., 2000), and, conversely, OPG deficient mice exhibit severe osteoporosis (Mizuno et al., 1998). Administration of RANKL to mice causes osteoporosis (Lacey et al., 1998), whereas disruption of the RANKL gene in mice leads to severe osteopetrosis, impaired tooth eruption, and the absence of osteoclasts (Kong et al., 1999).

Recently, several studies revealed the expression of RANKL and OPG in periodontal disease. In GCF, the ratio of the concentration of RANKL to that of OPG

was found significantly higher for periodontal disease patients than for healthy subjects (Mogi et al., 2004). Additionally, higher levels of RANKL protein and mRNA in periodontitis tissue than healthy tissue were observed and associated with lymphocyte and macrophage. In contrast, the levels OPG protein and mRNA were lower than that in the healthy group (Crotti et al., 2003; Liu et al., 2003). These evident supported the important role of the balance between RANKL and OPG in bone resorption of periodontitis.

Problems and hypothesis

A. actinomycetemcomitans is one of the periodontal pathogens that initiate the progression of periodontal disease resulting in both soft and hard tissue destruction MMPs and osteoclasts play role in these processes. Few studies focus on the correlation of *A. actinomycetemcomitans,* MMPs and osteoclastogenesis.

We hypothesized that production and LPS of *A. actinomycetemcomitans* could activate MMP-2 from human periodontal ligament (PDL) cells and, in particular, *A. actinomycetemcomitans* LPS could modify the balance of RANKL and OPG in PDL cells.

Specific aims

The specific aims of this study were:

1. To investigate the mechanism of supernatant from *A. actinomycetemcomitans* and *P. gingivalis* in MMP-2 activation which involves the balance of MT1-MMP and TIMP-2.

2. To investigate the mechanism of LPS from *A. actinomycetemcomitans* in MMP-2 activation.
3. To investigate the mechanism of LPS from *A. actinomycetemcomitans* in RANKL-OPG expression.

The study was performed in two approaches. First, PDL cells were stimulated with *A. actinomycetemcomitans* supernatant, MMP-2 activation and related molecules, MT1-MMP and TIMP-2 at both mRNA and protein level, were investigated. Second, in the same way, PDL cells were stimulated with *A. actinomycetemcomitans* LPS, MMP-2 activation, additionally, RANKL and OPG at mRNA and protein level were examined. Various inhibitors were used to study the mechanism of MMP-2 activation and signal transduction of the stimulation.

CHAPTER 2

MMP-2 ACTIVATION BY *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* SUPERNATANT IN HUMAN PDL CELLS WAS CORRESPONDED WITH REDUCTION OF 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 with 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 (Woolley 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., 1994b; Makela et al., 1994; Ingman et al., 1996; Korostoff et al., 2000b).

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 proteases such as plasmin or furin (Nagase, 1997; Sternlicht and Werb, 2001). However, these serine proteases 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 type1-matrix metalloproteinase (MT1-MMP) by the facilitation of tissue inhibitor of matrix metalloproteinase (TIMP-2). Then, another molecule of MT1-MMP cleaves the pro-domain of pro-MMP-2 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.

Experimental Procedures

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, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml 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 W. 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

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

P. 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, Sparks, MD, USA) in tryptic soy broth (TSB, BBL Microbiology system, Becton Dickinson, Sparks, MD, USA) supplemented with 1.5% yeast extract (Difco, Sparks, MD, USA), 5 μg/ml hemin (Sigma Chemical Co., St. Louis, MO, USA) and 0.2 μg/ml menadione (Boehringer Mannheim GmbH, Indianapolis, IN, USA).

E. coli, *S. salivarius* or *F. nucleatum* from clinical isolation were kindly provided from Department of Microbiology, Faculty of dentistry, Chulalongkorn University. *E. coli* and *S. salivarius* were grown in tryptic soy broth at 37°C in air. *F .nucleatum* was grown in tryptic soy broth at 37°C in anaerobic condition.

All strains were grown until an optical density reached 0.6-0.7 at 660 nm. The bacterial cultures were centrifuged (10,000x 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, Napervile, IL, USA) at a density of 50,000 cells/ml/well and were allowed to attach for 16 hours. After silencing the cells with serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO, USA) overnight, the supernatant from bacteria

were added and incubated for another 48 hours. All treatments were conducted in a serum-free condition and an equal amount of bacterial broth was added to the control. 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 protease inhibitors for 30 minutes before the bacterial supernatant was added. The inhibitors used in the experiment included 10 μM phenanthroline, 5 ng/ml (0.25 TIU) aprotinin, 0.2 mM Phenylmethanesulfonyl fluoride (PMSF) and 50 μg/ml leupeptin. All inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

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). Briefly, after electrophoresis, the gel was gently shaken in a renaturing buffer (0.25% Triton-X-100) at room temperature for 30 minutes to remove SDS, and then incubated in a developing buffer (0.15M NaCl/ 10 mM $CaCl_2$/ 50mM Tris-HCL pH 7.5/ 0.1% Brij35) at 37°C for 20 hours. The gel was stained with 2.5% Coomasie brilliant blue in 30% methanol and 10% acetic acid. The latent and active MMP-2 can be detected as clear bands at positions 72 kDa and 62 kDa, respectively.

For cell extract zymography, cells were extracted with RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0). The amount of protein was determined by BCA™ protein assay (Pierce, Rockford, IL, USA). All

samples, 25 μg of total protein per lane, were subjected to electrophoresis as described above.

Reverse-transcription polymerase chain reaction (RT-PCR)

Cells were seeded in 6-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 hours, total cellular RNA was extracted with Trizol (Gibco BRL, Carlsbad, CA, USA) according to manufacturer's instructions. One μg of each RNA sample was converted to cDNA by a reverse transcription using an AMV (Avian myeloblastosis virus) reverse transcritptase (Promega, Madison, WI, USA) for 1.5 hours 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 MT1-MMP, TIMP-2 and GAPDH primers were:

MT1-MMP	forward	5' CATCGCTGCCATGCAGAAGT 3'
	reverse	5' GTCATCATCGGGCAGCAC 3'
TIMP-2	forward	5' GGAAGTGGACTCTGGAAACGACATT 3'
	reverse	5' CTCGATGTCGAGAAACTCCTGCTTG 3'
GAPDH	forward	5' TGAAGGTCGGAGTCAACGGAT 3'
	reverse	5' TCACACCCATGACGAACATGG 3'

The PCR was performed using Taq polymerase with a PCR volume of 25 μl. The reaction mixtures contained 25 pmol of primers and 1μl 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, Napervile, IL, USA) 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 (150mM 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., St. Louis, MO). 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 (Sigma Chemical Co., St. Louis, MO, USA). 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 minutes, followed by another 30 minutes staining with peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA). After extensive washes with PBS, the membrane was coated for the

chemiluminescence detection (Pierce, Rockford, IL, USA) for 1 minutes and the signal was captured with CL-Xposture film (Pierce, Rockford, IL, USA). The amount of protein was determined using a densitometer (Imaging densitometer GS-700, BioRad, Hercules, CA, USA) and Molecular Analyst Software (BioRad, Hercules, CA, USA).

Statistic 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 2.1A showed that the supernatant could activate MMP-2 (62 kDa) in a dose dependent manner with an optimal dose of about 50 µl/ml. 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.*

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 of *A. actinomycetemcomitans* or 10 µl/ml of *P. gingivalis* supernatant for 48 hours. 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 2.1B). 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 *E. coli*, *S. salivarius* and *F. nucleatum* did not contain the ability of MMP-2 activation in HPDL cells (Figure 2.2) that also indicates the specificity of the supernatant from *A. actinomycetemcomitans* in MMP-2 activation.

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 minutes prior to the *A. actinomycetemcomitans* supernatant and followed by another 48-hour 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 2.3A. 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 2.3B).

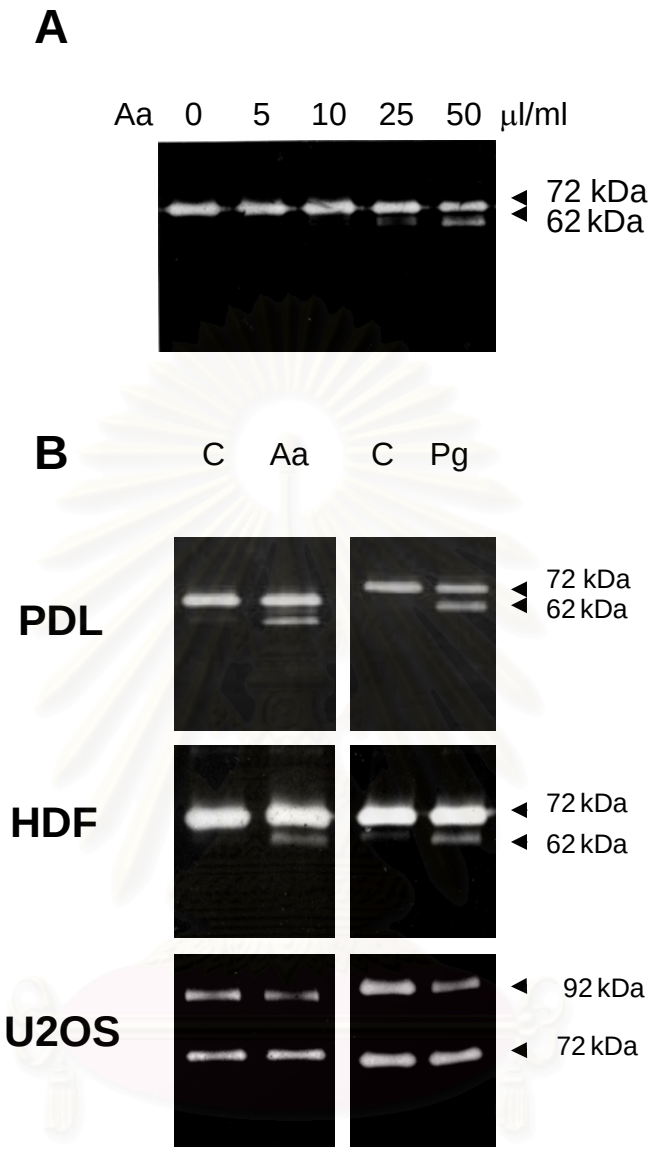


Figure 2.1 (**A)** HPDL cells were treated with 0, 5, 10, 25, 50 μl/ml of supernatant for 48 hours and the activation of MMP-2 was analyzed by gelatin zymography. The activation could be detected clearly when treated with 50 μl/ml. **(B)** HPDL cells, human dermal fibroblast (HDF) and human osteosarcoma cell line (U2OS) were treated with either 10 μl/ml of *P. gingivalis* supernatant or 50 μl/ml of *A. actinomycetemcomitans* supernatant for 48 hours and conducted the gelatin zymography analysis. 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.

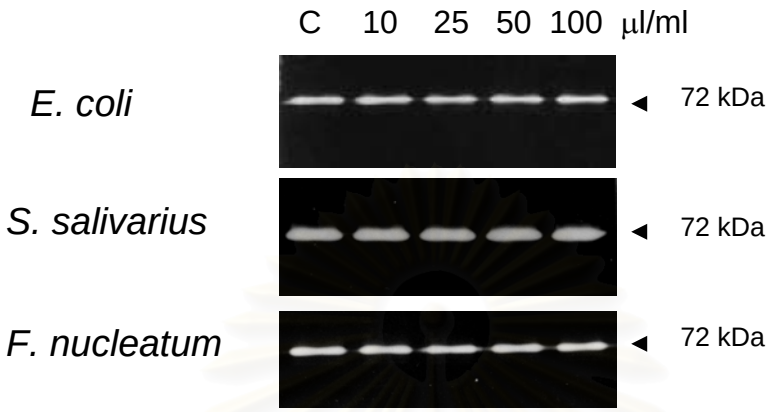


Figure 2.2 HPDL cells were treated with 0, 10, 25, 50, 100 μl/ml of either *E. coli*, *S. salivarius* or *F. nucleatum* supernatant for 48 hours and the activation of MMP-2 was analyzed by gelatin zymography. An equal amount of bacterial broth was added to the control. No active MMP-2 was detected in PDL cells treated with these bacterial supernatant. Positions of the latent MMP-2 (72 kDa) are indicated on the right.

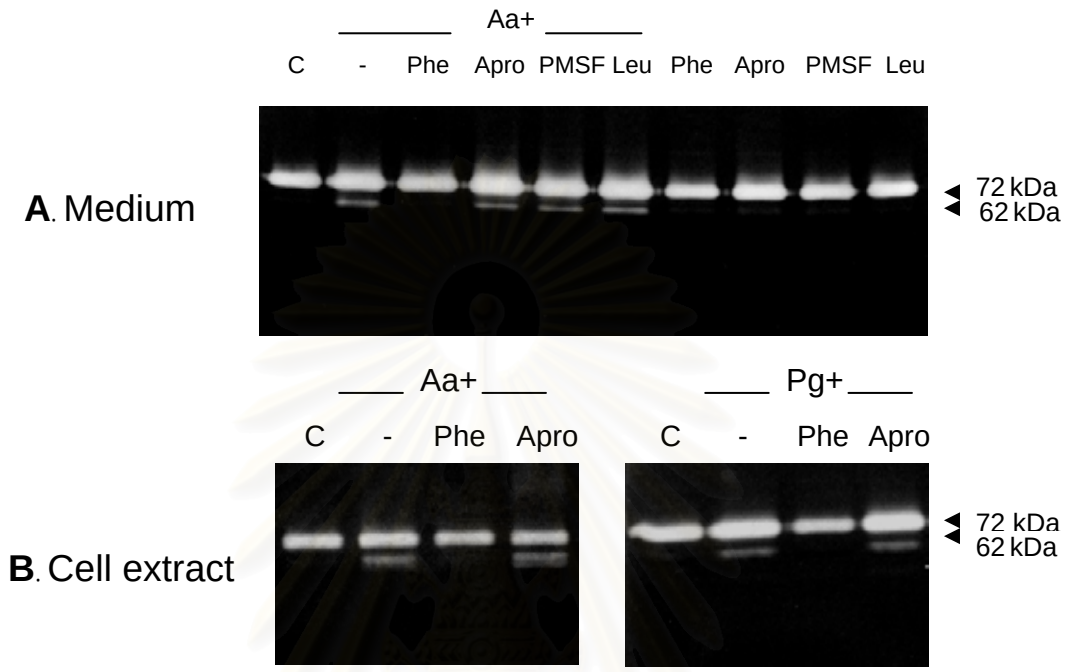


Figure 2.3 (A) Gelatin zymography shows the inhibitory effect of protease inhibitors. HPDL cells were treated with 50 μl/ml of *A. actinomycetemcomitans* (Aa) supernatant in the presence or absence of protease inhibitors; phenanthroline (Phe), aprotinin (Apro), PMSF and leupeptin (Leu). The figure shows that the activation of MMP-2 could be inhibited only by phenanthroline. Inhibitors alone did not have any effects on the activation. Fifty μl/ml of BHI was added to the control (c). (**B)** Gelatin zymography shows that 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.

***A. 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 analyses, respectively. The results shown in Figure 2.4 demonstrated that the processes of MMP-2 activation by *A. actinomycetemcomitans* and *P. gingivalis* supernatant were different even though the supernatant from both types of bacteria could exert MMP-2 activation.

In Western blot analysis (Figure 2.4A), 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 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 2.4B 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 2.5 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 2.5A) while the mRNA level increased about 2.5 fold (one way ANOVA, Scheffe's test $p<0.05$) (Figure 2.5B). 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 one fold from the control ($p<0.05$) (Figure 2.5A).

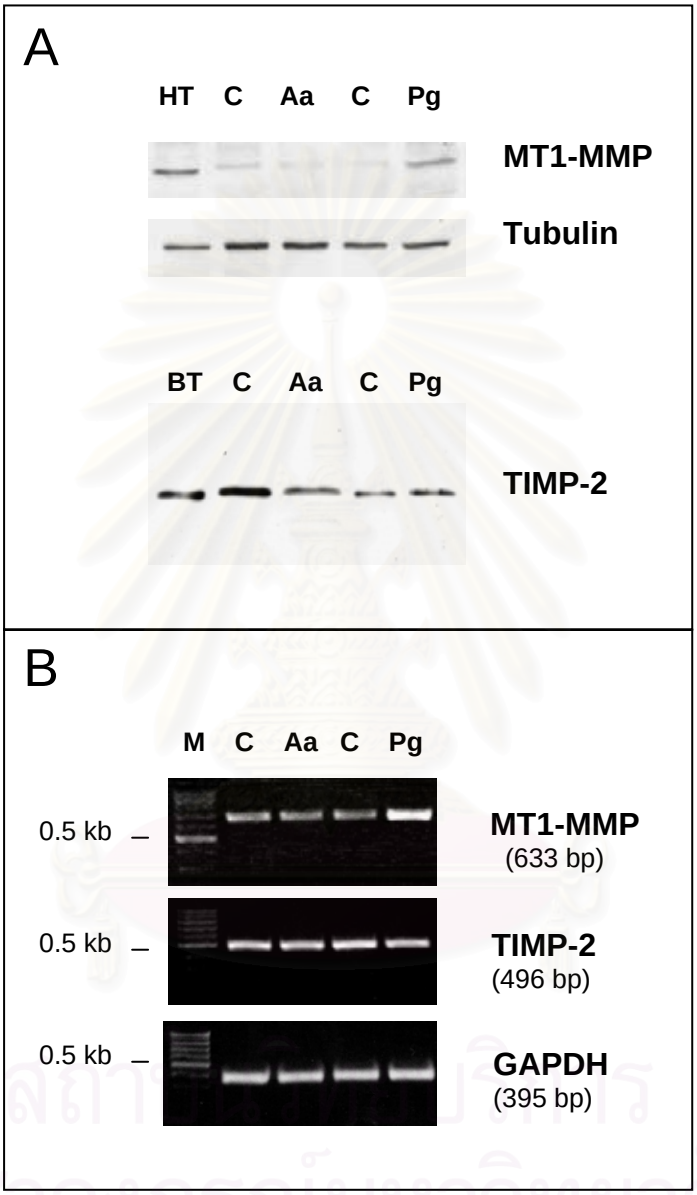

A
HT C Aa C Pg
MT1-MMP
Tubulin
BT C Aa C Pg
TIMP-2
B
M C Aa C Pg
0.5 kb
MT1-MMP
(633 bp)
0.5 kb
TIMP-2
(496 bp)
0.5 kb
GAPDH
(395 bp)

Figure 2.4

Figure 2.4 (A) shows 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)** shows 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.

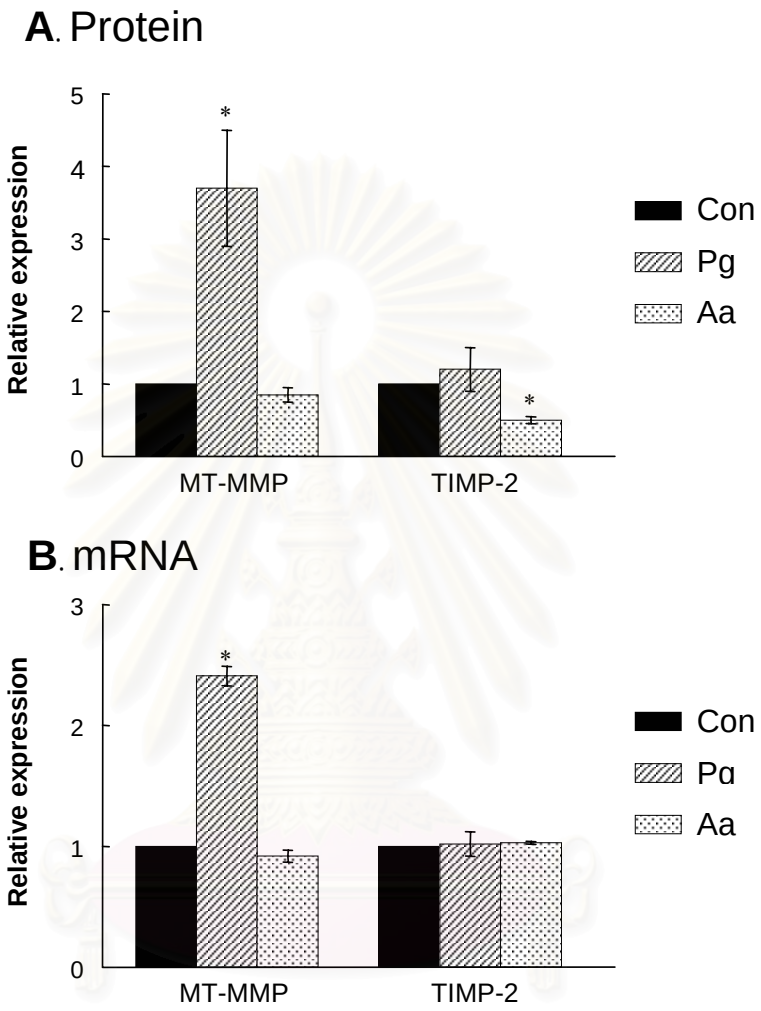


Figure 2.5 The histograms summarized the change of MT1-MMP and TIMP-2 from three different experiments. **(A)** shows the protein level while **(B)** shows the mRNA level of MT1-MMP and TIMP-2. The graph marked with asterisk denotes any significant differences when compared with its control (one way 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

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. 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 protease 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 the 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, since 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.

CHAPTER 3

ACTINOBACILLUS ACTINOMYCETEMCOMITANS LIPOPOLYSACCHARIDE ACTIVATES MATRIX METALLOPROTEINASE-2 AND INCREASES RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS

Introduction

In periodontitis, certain species of Gram-negative bacteria harbored in periodontal pockets play a major role in the pathogenesis of the disease (Slots and Listgarten, 1988; Haffajee and Socransky, 1994). Among these bacteria, *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) has been implicated as an etiological agent in juvenile and adult periodontits (Zambon et al., 1986; Slots and Listgarten, 1988). *Actinobacillus actinomycetemcomitans* produces a multitude of products that participate in the process of inflammation and tissue destruction. Among these products, the LPS of *A. actinomycetemcomitans* has been considered to be involved in the pathogenesis of alveolar bone loss and connective tissue degradation in periodontal disease (Ueda et al., 1998).

Lipopolysaccharide was known to be an inducer of bone resorption by enhancing osteoclastogenesis (Zou and Bar-Shavit, 2002). Although it is still not clear whether LPS could directly target osteoclast precursors, many studies provided the evidence that LPS indirectly influenced the osteoclast formation through osteoblasts or bone marrow stromal cells (Pelt et al., 2002; Zou and Bar-Shavit, 2002). In addition, LPS is also known able to induce the secretion of several inflammatory

cytokines (Saglie et al., 1990; Kesavalu et al., 2002). Increasing of these cytokines causes the imbalance of proteolytic enzymes which eventually leads to connective tissue and bone destruction (Schwartz et al., 1997; Siwik et al., 2000).

A family of proteolytic enzymes, namely matrix metalloproteinases or MMPs, has been considered to play roles in connective tissue degradation in both physiological and pathological situations (Birkedal-Hansen, 1993; Shapiro, 1998). A number of MMPs have been detected in the crevicular fluid of patients with periodontitis and are believed to play an important role in periodontal tissue degradation (Birkedal-Hansen, 1993). These MMPs include the interstitial collagenase such as MMP-1, MMP-8, stromelysin (MMP-3) and MMP-9. Matrix metalloproteinase-2, a 72-kDa gelatinase, was recently shown to be involved in the pathogenesis of periodontal disease (Makela et al., 1994; Korostoff et al., 2000b). The amount of both latent and active MMP-2 was found to increase in the periodontal tissue of patients with periodontitis. Matrix metalloproteinase-2 is an enzyme that is secreted mainly from fibroblasts, including human gingival fibroblasts (HGF) and human periodontal ligament (HPDL) cells (Lapp et al., 2003; Pattamapun et al., 2003). The enzyme is secreted in latent form and requires activation for proper function. In our previous study, we found that the supernatant from *Porphyromonas gingivalis (P. gingivalis)*, another etiological bacterium in periodontitis, could induce the activation of MMP-2 by increasing the expression of membrane-type matrix metalloproteinase 1 (MT1-MMP) in HPDL cells (Pattamapun et al., 2003). These results suggested the role of MMP-2 and HPDL cells in the process of connective tissue degradation in periodontal disease.

HPDL cells may also play a role in osteoclastogenesis through the expression of receptor activator of NF-kB ligand (RANKL) on their cell surfaces (Hasegawa et al., 2002; Crotti et al., 2003; Liu et al., 2003). It is widely accepted that formation of osteoclasts requires the interaction between receptor activator of NF-kB or RANK, which is expressed on the surface of osteoclasts and its ligand, RANKL, in the presence of macrophage colony-stimulating factor (M-CSF) (Suda et al., 1999). RANKL is found on the surface of many cell types, including osteoblasts and bone marrow stromal cells. Expression of RANKL in HPDL cells leads to a hypothesis that they play a role in osteoclast formation. In addition, HPDL cells secrete a certain amount of osteoprotegerin (OPG), a decoy tumor necrosis factor (TNF) receptor, which is able to bind to RANKL (Hasegawa et al., 2002). An interaction between OPG and RANKL will inhibit the formation of osteoclast. Hence, a balance of RANKL and OPG in HPDL cells may associate with the homeostasis of the periodontium.

The purpose of this study was to examine the effect of the LPS of *A. actinomycetemcomitans* in HPDL cells. The study was to focus on an ability of the LPS from *A. actinomycetemcomitans* on the activation of MMP-2 and the alteration of RANKL and OPG, which were the factors involved in connective tissue degradation and bone resorption.

Experimental Procedures

Cell culture

Human PDL 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. Briefly, teeth were rinsed with sterile phosphate buffer saline several times and the PDL were scraped out from the middle third of the root. The explants were harvested on a 60-mm culture dish (Nunc, Naperville, IL, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B (Gibco BRL, Carlsbad, CA, USA) at 37 $^{\circ}$C in humidified atmosphere of 95% air, 5% CO_2. Cells from the third to the fifth passage were used.

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

Preparation of Lipopolysaccharide

Actinobacillus actinomycetemcomitans (ATCC 43718) was cultivated in brain heart infusion broth (BHI, Difco, Sparks, MD, USA) at 37°C, 5% CO_2. The LPS was prepared by water-phenol extraction as described by Westphal and Jann (1966). The amount of the LPS used in the experiment was determined by dry weight.

Activation of HPDL cells with the LPS

Human PDL cells were seeded in 6-well plates (Nunc, Naperville, IL, USA) at a density of 25,000 cells/cm^2 and were allowed to attach for 16 hours. After silencing the cells with serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO, USA) overnight, the LPS of *A. actinomycetemcomitans* was added and incubated for another 36 hours. All treatments were conducted in a serum-free condition. After 36 hours, the medium was collected and kept at –20 °C prior to an analysis.

In the inhibitory experiment, cells were treated with inhibitors for 30 minutes prior to the treatment of bacterial LPS. The inhibitors used in the experiment included 10 μM Phenanthroline, 5 ng/ml (0.25 TIU) Aprotinin, 50 μM Pyrrolidinedithiocarbamate ammonium salt (PTDC) and 1 μM Indomethacin. All inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO, USA). To verify the involvement of human lipopolysaccharide binding protein (LBP), the inhibitory antibody against LBP, cloned 6G3 (HyCult biotechnology b.v., Uden, the Netherlands), was used.

Gelatin zymography

The activity of MMP-2 was evaluated by gelatin zymography. The medium was subjected to a 10% SDS-polyacrylamide gel containing 0.1% gelatin under a non-reducing condition. After electrophoresis, the gel was gently shaken in a renaturing buffer (0.25% Triton-X-100) at room temperature for 30 minutes to remove SDS, and then incubated in a developing buffer (0.15M NaCl/ 10 mM $CaCl_2$/ 50mM Tris-HCL pH 7.5/ 0.1% Brij35) at 37°C for 20 hours. The gel was stained with 2.5% Coomasie

brilliant blue in 30% methanol and 10% acetic acid. The latent and active MMP-2 can be detected as clear bands at the positions 72 kDa and 62 kDa, respectively.

Reverse-transcription polymerase chain reaction (RT-PCR)

Cells were treated with the LPS of *A. actinomycetemcomitans* as described above for 36 hours. Total cellular RNA was extracted with Trizol (Gibco BRL, Carlsbad, CA, USA) according to manufacturer's instructions. One μg of each RNA sample was converted to cDNA by a reverse transcription using an AMV (Avian myeloblastosis virus) reverse transcriptase (Promega, Madison, WI, USA) for 1.5 hours at 42°C. Subsequent to the reverse transcription, a polymerase-chain reaction was performed. The primers were prepared (Genset Biotech, Singapore) following the reported sequences from GenBank (MT1-MMP;GI:4826833, TIMP-2;GI:9257247, RANKL;GI;2612921, OPG;GI:2072184, COX-2;GI:181253 GAPDH;GI:4503912). The oligonucleotide sequences of the primers were:

MT1-MMP	forward	5' CATCGCTGCCATGCAGAAGT 3'
	reverse	5' GTCATCATCGGGCAGCAC 3'
TIMP-2	forward	5' GGAAGTGGACTCTGGAAACGACATT 3'
	reverse	5' CTCGATGTCGAGAAACTCCTGCTTG 3'
RANKL	forward	5' CCAGCATCAAAATCCCAAGT 3'
	reverse	5' CCCCTTCAGATGATCCTTC 3'
OPG	forward	5' TGCAGTACGTCAAGCAGGAG 3'
	reverse	5' TGACCTCTGTGAAAACAGC 3'
COX-2	forward	5' TTC AAA TGA GAT TGT GGG AAA GCT 3'
	reverse	5' AGA TCA TCT CTG CCT GAG TAT CTT 3'

GAPDH forward 5’ TGAAGGTCGGAGTCAACGGAT 3’

reverse 5’ TCACACCCATGACGAACATGG 3’

The PCR was performed using Taq polymerase with a PCR volume of 25 μl. The mixtures contained 25 pmol of primers and 1μl 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

RANKL and tubulin were analyzed from cell extracts using RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing cocktail protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). The amount of protein was determined by 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 membrane was stained with antibody for RANKL (AB1862, Chemicon Temecula, CA, USA) or anti-tubulin antibody (a gift from Professor Erik Thompson, St. Vincent’s Institute of Medical Research, Melbourne, Victoria, Australia). 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 minutes, followed by another 30 minutes staining with peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA). The membrane was coated with chemiluminescence reagent (Pierce, Rockford,

IL, USA) and the signals were captured with CL-Xposture film (Pierce, Rockford, IL, USA). The amount of protein was determined using a densitometer (Imaging densitometer GS-700, BioRad, Hercules, CA, USA) and Molecular Analyst Software (BioRad, Hercules, CA, USA).

Results

The result in Figure 3.1A demonstrated the effect of the LPS prepared from *A. actinomycetemcomitans* on the activation of MMP-2 in HPDL cells. The activation of MMP-2 could be identified by the presence of a 62-kDa band in the zymography. Lipopolysaccharide of *A. actinomycetemcomitans* activated MMP-2 in a dose dependent manner, starting from 1 μg/ml of the LPS. However, no changes in the total level of the enzyme could be observed when analyzed with a densitometer. The experiment was performed triplicate using HPDL cells from three donors.

Human PDL cells were activated with 2 μg/ml of the LPS of *A. actinomycetemcomitans* in the presence of protease inhibitors, Aprotinin, a serine protease inhibitor, and Phenanthroline, an MMP inhibitor. The result in Figure 3.1B showed the disappearance of a 62-kDa band in lane 3 (+ LPS, + Apro) as compared to lane 2 (+LPS), indicated that Aprotinin could inhibit the effect of the LPS on the activation of MMP-2. On the contrary, no changes of the activation could be observed in the presence of Phenanthroline (compared lane 2 with 4). In addition, Pyrrolidinedithiocarbamate (PTDC), an NF-kB inhibitor, could also reverse the effect of the LPS on the activation (lane 5), suggesting the involvement of NF-kB in the mechanism of activation by the LPS of *A. actinomycetemcomitans*.

Figure 3.2 showed RT-PCR analysis of the expression of MT1-MMP and TIMP-2 after treatment with the LPS of *A. actinomycetemcomitans* for 36 hours. No change of the expression of MT1-MMP and TIMP-2 was found when compared to the control. The results corresponded with those from the inhibitory experiment, which demonstrated that Phenanthroline could not inhibit the activation of MMP-2 induced by the LPS of *A. actinomycetemcomitans* and the mechanism of the activation was not an MMP-dependent pathway.

The expression of RANKL and OPG after treatment with the LPS of *A. actinomycetemcomitans* was shown in Figure 3.3. The increase of RANKL expression was observed, while the level of OPG expression remained the same. We thus further examined the effect of the LPS on the expression of RANKL using Western blot analysis as shown in Figure 3.4. The similar result was obtained since the amount of RANKL increased as compared to the control.

Addition of Aprotinin to the culture medium could prevent the up-regulation of RANKL induced by the LPS of *A. actinomycetemcomitans.* (Figure 3.5) The results also revealed that the effect of the LPS on RANKL was abolished by Indomethacin, a non-specific COX inhibitor. The inhibitory effect of Indomethacin suggested the COX pathway in the mechanism of RANKL induction.

Figure 3.6A revealed the up-regulation of COX-2 in HPDL cells after treatment with the LPS. It is interesting to note that application of Aprotinin could inhibit an increase of COX-2 that was induced by the LPS of *A. actinomycetemcomitans.* The results suggested that the initial signal of LPS-induced RANKL in HPDL cells might be an activity of serine protease.

To clarify whether LBP remains in our serum-free system, we added the neutralized antibody to LBP to the medium before treatment with the LPS (Figure 3.6B). Addition of the anti-LBP did not inhibit the effect of the LPS on RANKL suggesting that the response of HPDL cells to the LPS might not involve in LBP-CD14 signaling pathway.

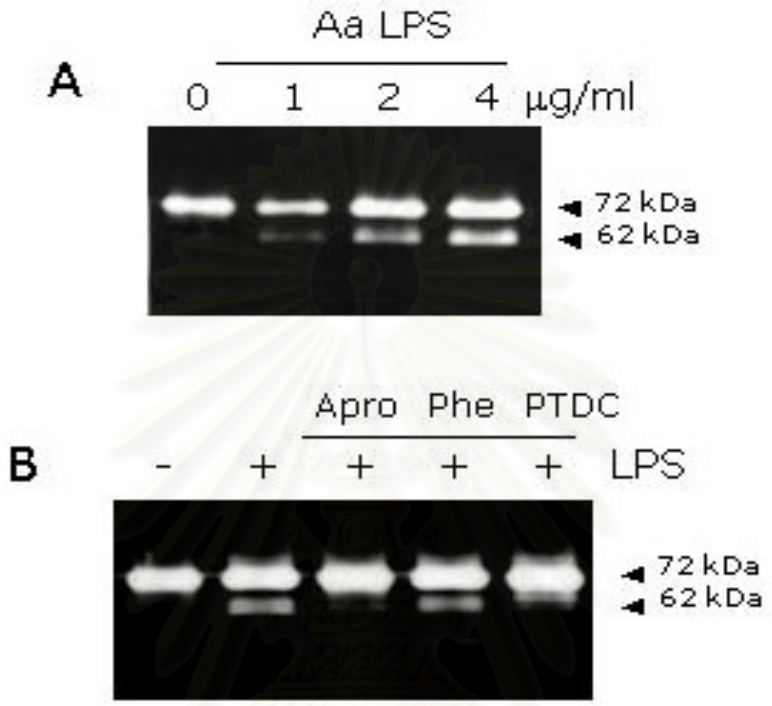


Figure 3.1 (A) Gelatin zymography demonstrates the effect of LPS from *A. actinomycetemcomitans* on the activation of MMP-2. Addition of the LPS to the culture of HPDL cells induces the activation of MMP-2 in a dose dependent manner. **(B)** shows the inhibitory effect of Aprotinin (Apro) and Pyrrolidinediethiocarbamate (PTDC) on the activation of MMP-2 induced by the LPS (+LPS), while Phenanthroline (Phe) does not show the inhibitory effect on the activation. The arrowheads indicate the positions of latent MMP-2 (72kDa) and active MMP-2 (62 kDa). The results represent one of the triplicate experiments.

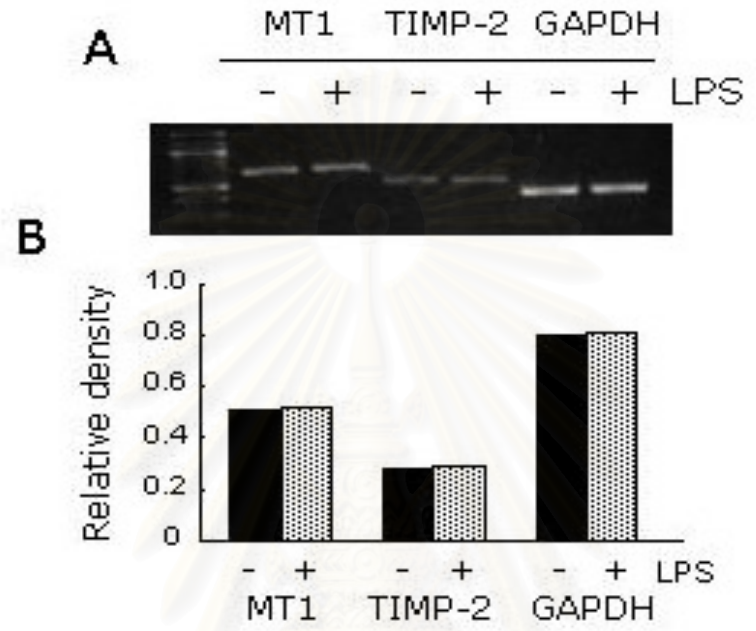


Figure 3.2 An analysis of MT1-MMP and TIMP-2 by RT-PCR. **(A)** shows the level of MT1-MMP (MT1) and the expression of TIMP-2 in HPDL cells after treatment with 4 μg/ml of the LPS of *A. actinomycetemcomitans* (+LPS). No change of the levels of MT1-MMP or TIMP-2 is observed as compared to GAPDH. **(B)** indicates a relative density of the PCR product in (A). The figure represents one of three experiments.

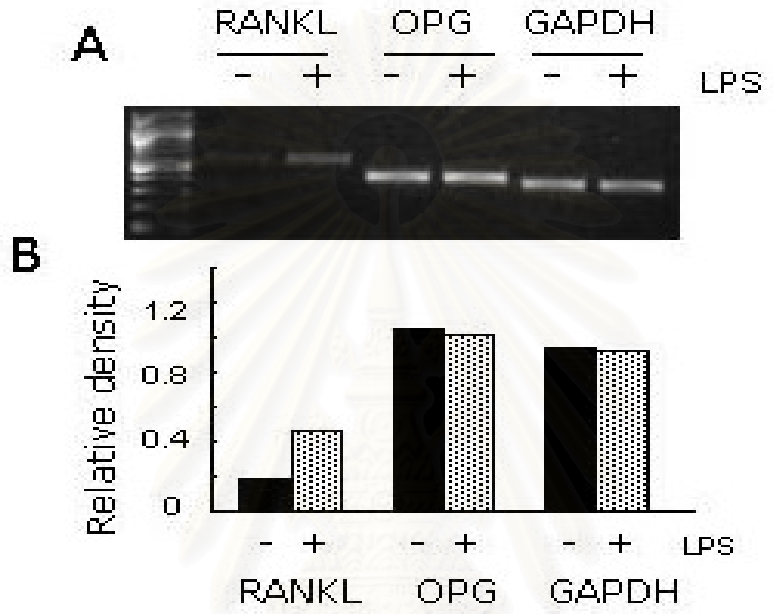


Figure 3.3 An analysis of RANKL and OPG by RT-PCR. **(A)** reveals the expression of RANKL and OPG in HPDL cells after treatment with 4 μg/ml of the LPS (+LPS). The relative density of the PCR product is shown in **(B)**. The LPS increases the expression of RANKL but not OPG. The figure represents one of the triplicate experiments.

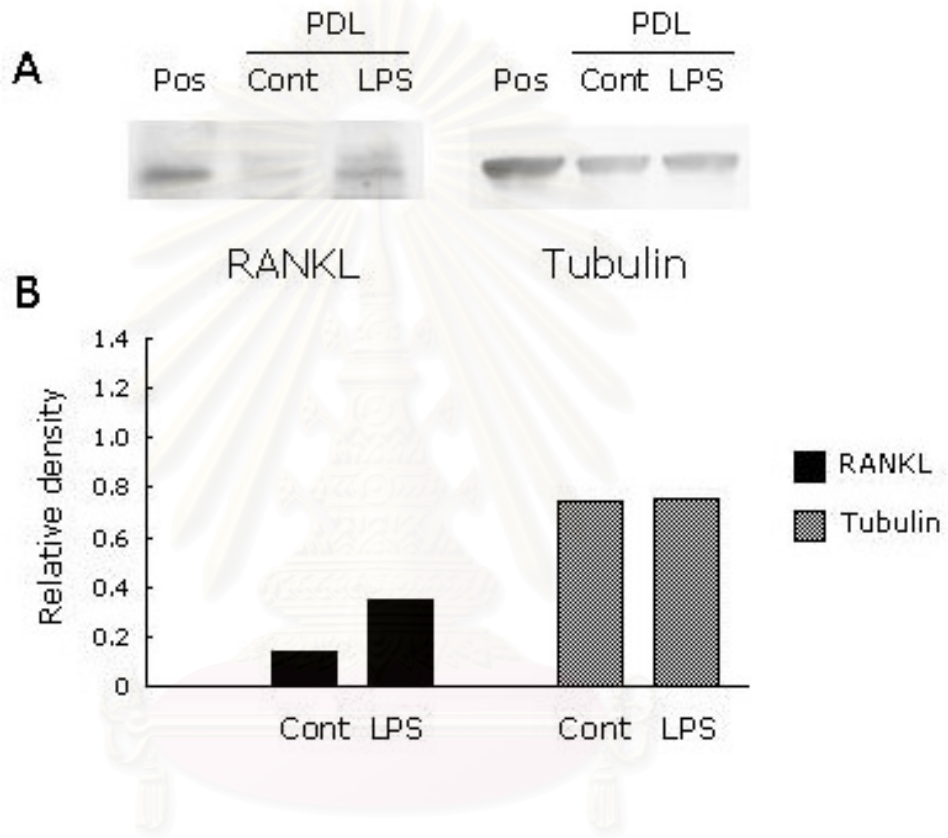


Figure 3.4 Western blot analysis of the expression of RANKL on HPDL cells after treatment with the LPS of *A. actinomycetemcomitans* **(A)**. The protein extracted from SaOS-2 is used as positive control (Pos). The relative density of RANKL is shown in **(B)**. Tubulin is used as internal control. The results represent one of the triplicate experiments.

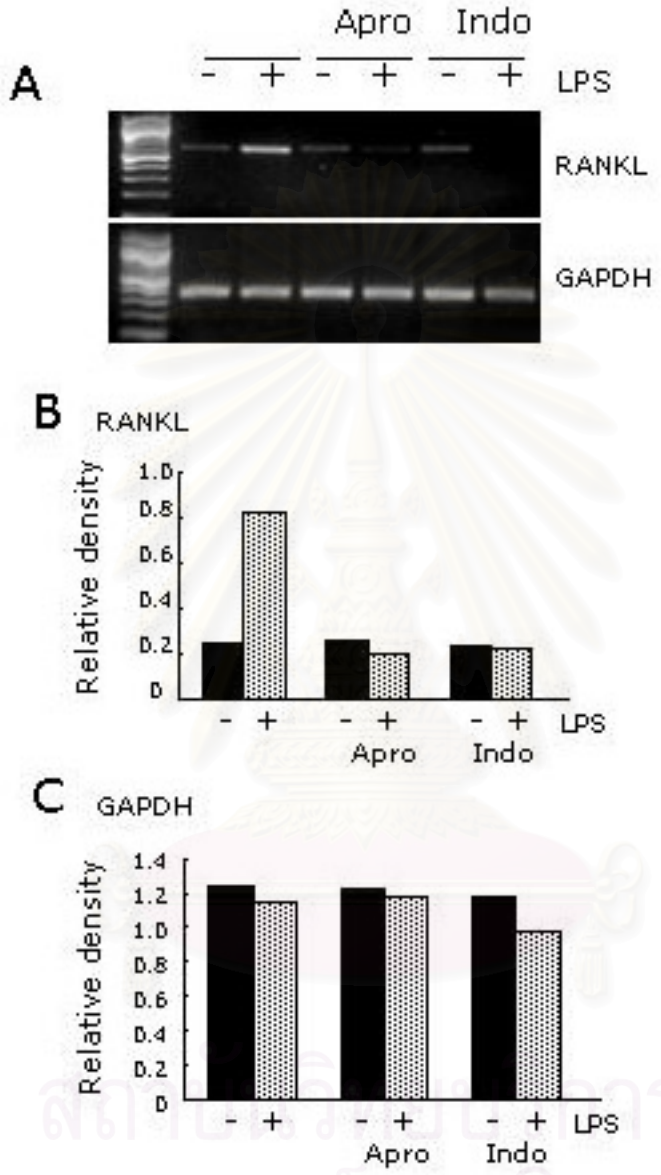


Figure 3.5 RT-PCR analysis shows the effect of Aprotinin (Apro) and Indomethacin (Indo) on RANKL expression after treatment with the LPS of *A. actinomycetemcomitans*. Both Aprotinin and Indomethacin inhibit the inductive effect of LPS on RANKL expression **(A)**. **(B)** and **(C)** show the relative density of PCR products of RANKL and GAPDH in (A), respectively.

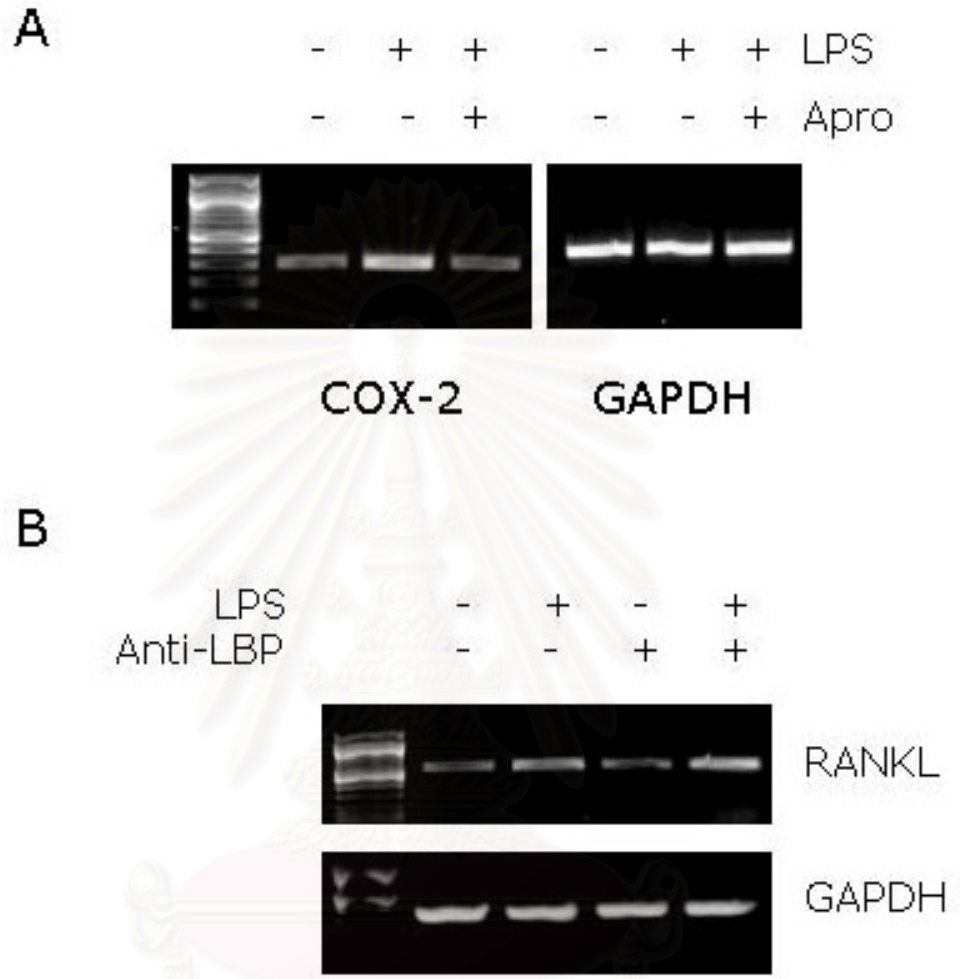


Figure 3.6 **(A)** RT-PCR analysis indicates the inhibitory effect of Aprotinin (Apro) on the expression of COX-2 induced by the LPS. **(B)** RT-PCR analysis of RANKL expression in HPDL cells induced by the LPS in the presence or absence of inhibitory antibody against human LPS binding protein (anti-LBP).

Discussion

We found in this study that HPDL cells could respond directly to the LPS of *A. actinomycatemcomitans*. In general, LPS binds to a cell surface protein, CD14, with the help of LPS binding protein (LBP), which is present in the serum. A trans-membrane signaling is then initiated by the Toll-like receptor (TLR), which has been shown to link to LPS/LBP/CD14 complex (Gegner et al., 1995; Yang et al., 1998). Due to the requirement for LBP, CD14-mediated pathways are thus serum-dependent. However, we used serum-free condition in this study, indicating that the response of HPDL cells might not occur through CD14. This notion is further supported by addition of neutralizing antibody to LBP to the medium. The increase of RANKL is still observed in the presence of the neutralizing antibody suggesting the different mechanism other than the LBP-CD14 signaling pathway. Kim and Koh (2000) also reported the similar results in endothelial cells, when they treated endothelial cells with LPS in serum-free condition. The exact mechanism of the response of HPDL cells to LPS remains to be elucidated.

Lipopolysaccharides can induce MMP-2 activation in HPDL cells. Recently, Korostoff et al. (2000) reported that the level of active MMP-2 increased in the periodontal tissue of patients with periodontal disease. The function of MMP-2 involves in the degradation of type IV collagen in the basement membrane and denatured type I collagen. The activation of this enzyme is correlated to invasiveness of several cancer cells, since the metastasis requires an ability of cancer cells to destroy the basement membrane (Ellerbroek and Stack, 1999). Lately, MMP-2 has been shown to degrade native type I collagen and has been considered as a major enzyme involved in the turnover of soft connective tissue (Creemers et al., 1998). An

increase of active MMP-2 found in periodontitis suggests the role of the enzyme in the degradation of periodontium.

We have also tested the effect of LPS from *Fusobacterium nucleatum* (*F. nucleatum*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) in order to investigate the specificity of the LPS from *A. actinomycetemcomitans* (data not shown). *Fusobacterium nucleatum* and *P. aeruginosa* are Gram-negative bacteria found in dental plaque and in soil and water, respectively. The results revealed that the LPS from *F. nucleatum* and *P. aeruginosa* also activated MMP-2 and increased RANKL expression. Our results indicate that the ability of LPS to activate MMP-2 and to induce RANKL expression is not limited to certain species of the bacterium. The ability might be a characteristic of LPS from several species of Gram- negative bacteria. The result is in concurrence with the report from others (Kim and Koh, 2000) demonstrating that the LPS of *Escherichia coli* (*E. coli*) caused the activation of MMP-2.

Our previous study (Pattamapun et al., 2003) showed that HPDL cells responded to the supernatant from *P. gingivalis* by increasing the level of active MMP-2 and MT1-MMP. MT1-MMP is a membrane-bounded MMP that function not only in degradation of the tissue but also in a process of MMP-2 activation. The up-regulation of MT-MMP has been shown to correspond with the activation of MMP-2 (Philip et al., 2001; Sounni et al., 2002). However, we found that Phenanthroline, a zinc chelator that acts as an MMP inhibitor, could not inhibit the activation by LPS of *A. actinomycetemcomitans*, suggesting that the process of activation might be different from the mechanism described above. An inhibition by Aprotinin suggested that the mechanism might occur through a serine protease-dependent pathway. Our

finding is similar to the report by Takeda et al. (Takeda et al., 2000) who found that LPS of *E. coli* contained an activity of serine protease to activate MMP-2 in cervical fibroblasts.

Since the structure of LPS is typically consisting of a hydrophobic domain or lipid-A, a core oligosaccharide and a distal polysaccharide or O-antigen, the presence of protease activity in LPS is not corresponded with its structure. However, it is possible that LPS might induce the activity of serine protease from HPDL cells, which involves in the process of MMP-2 activation. Generally, activation of MMPs requires a function of serine protease such as plasmin (Murphy et al., 1999) but that of MMP-2 requires different molecules from others. An accepted model of MMP-2 activation is an MMP-dependent pathway. The model was proposed by Strongin et al. (Strongin et al., 1995), which described the formation of tri-molecular complex of MMP-2, MT1-MMP and TIMP-2 as an initial step of the activation. The pro-domain of MMP-2 is then cleaved by another MT1-MMP molecule to yield a 68-kDa intermediate form of MMP-2, which is further processed into a 62-kDa, active MMP-2. However, there are a few reports describing the function of serine protease in MMP-2 activation, for example, the activation of MMP-2 in astrocytes from glioma by plasminogen activator or by proteinase-3, a serine protease from polymorphonuclear leukocytes (Le et al., 2003; Pezzato et al., 2003). The evidence suggests the possible action of serine protease in the activation of MMP-2.

In addition, our results revealed that PTDC, an NF-kB inhibitor, could inhibit the activation of MMP-2 induced by the LPS of *A. actinomycetemcomitans*. The NF-kB has been shown to involve in the regulation of both serine proteases and MMPs (Kim and Koh, 2000; Philip et al., 2001; Sliva et al., 2002). The function of NF-kB

also involves in MMP-2 activation through the function of MT1-MMP (Kim and Koh, 2000; Philip et al., 2001). In addition, it has been reported that LPS could induce a production of inflammatory cytokines through an NF-kB-dependent pathway (Yoza et al., 1996), which might affect the activation of MMP-2. However, the exact role of NF-kB in the activation of MMP-2 in HPDL cells induced by the LPS needs further investigation.

Apart from the function of MMP-2 activation, we also found that the LPS of *A. actinomecetemcomitans* induced the expression of RANKL in HPDL cells. RANKL is a surface protein that plays an important role in osteoclast formation. Generally, LPS has been considered to be a potent inducer of osteoclast formation. It has been shown that LPS induced RANKL expression in osteoblasts and marrow stromal cells (Zou and Bar-Shavit, 2002). The increase of RANKL in these cells may provide a support for osteoclastogenesis and results in the increased number of osteoclasts *in vitro.*

Recent reports revealed that the expression of RANKL increased in the gingival tissue of patients with periodontitis (Crotti et al., 2003; Liu et al., 2003). The increase of RANKL corresponds with that of bone resorption, which is a major characteristic of periodontal disease, suggesting the role of RANK in pathogenesis of periodontitis. Furthermore, the function of RANKL is not limited only to osteoclastogenesis but also involves in activation and survival of osteoclasts via a binding between RANK and RANKL (Takami et al., 1999; Udagawa et al., 1999). Thus, an increase of RANKL induced by the LPS in HPDL cells might be significant in the process of alveolar bone resorption in periodontitis.

Expression of several factors, such as IL-1β, IL-11, TNF-α and prostaglandin E2, have been shown to involve in the expression of RANKL in bone and marrow stromal cells (Yasuda et al., 1998; Suda et al., 1999; Hofbauer et al., 1999b). In PDL cells, Kanzaki et al. (2002) found that application of mechanical stress to PDL cells *in vitro* increased the expression of RANKL through PGE_2 synthesis. The similar mechanism might occur in this study since Indomethacin exerted the effect on blocking an elevation of RANKL in the LPS-treated HPDL cells.

Application of Aprotinin could block the effect of the LPS of *A. actinomycetemcomitans* in RANKL induction. Since Aprotinin could inhibit both MMP-2 activation and RANKL induction, it is possible that the serine protease that involves in the activation of MMP-2 plays a role in the induction of RANKL. Taking into account that Aprotinin could inhibit COX-2 expression induced by the LPS, suggesting that the action of serine protease might be responsible for both the activation of MMP-2 and the up-regulation of RANKL in HPDL cells. A mechanism of the activity of serine protease, either from the LPS itself or from the LPS-induced HPDL cells, is still unclear.

In conclusion, we reported here that the LPS of *A. actinomycetemcomitans* could induce the activation of MMP-2 and the expression of RANKL in HPDL cells. The induction might be involved by the activity of serine protease. This finding supports that HPDL cells can respond directly to the LPS of *A. actinomycetemcomitans* and participate in the destruction of periodontium.

CHAPTER 4

GENERAL DISCUSSIONS AND FUTURE STUDIES

Pathogenesis of the periodontal diseases

Major features of periodontitis are destruction of periodontal ligament, resorption of alveolar bone, and formation of periodontal pockets. These destructions are mainly a consequence of bacteria-induced host response. Cells in the periodontium, including gingival and periodontal ligament fibroblasts, epithelial cells, inflammatory cells (neutrophils, macrophages and mast cells) and bone cells (osteoblasts and osteoclasts) , are all responsible for these processes.

Periodontal pathogens have an ability to colonize the tooth surface in the gingival crevice by evading host defenses. These pathogens release a large number of products and metabolites including proteases and lipopolysaccharide, and also the bacteria cell itself that could invade periodontal tissue. The first line of defense to the bacterial challenges is gingival crevicular fluid (GCF) and junctional epithelial barrier. Beside of being directly damaged, epithelial cells lining the junctional epithelium are responded to the bacteria infection by secreting enzyme, MMP-9 (Smith et al., 2004), inflammatory mediators, IL-1 and IL-8 (Sfakianakis et al., 2001), cytokines, PDGF (Pinheiro et al., 2003), HGF (Hoshima et al., 2002), G-CSF, GM-CSF (Sugiyama et al., 2002), which believed to contribute to disease progression in periodontitis.

When the first line of defense is impaired by invading bacteria and their product, inflammatory reactions and acquired immune response are showed up. Although the periodontal pathogens possess potent mechanisms of evading or damaging host defenses, for example, direct damage of PMNs and macrophages by

A. actinomycetemcomitans leukotoxin (Tsai et al., 1979; Rabie et al., 1988), degradation of immunoglobulin (Jansen et al., 1995; Hollmann and Van der Hoeven, 1999; Bachrach et al., 2004) and degradation of periodontal tissue. The bacteria-induced host response is still the prominent process of the development of periodontitis.

Immune response to periodontal pathogen antigen generates two sides of reaction, host protection and tissue damage. Activation of humoral immunity leads to an accumulation of plasma cells and the production of immunoglobulins, which will activate the complement cascade and lead to inflammation and generation of prostaglandins. Stimulation of cellular immunity leads to the production of lymphokines from activated T-lymphocytes, which modulate macrophage activity. As a result, the inflammatory reaction is promoted.

Inflammatory cells participating in periodontitis include neutrophils polymorphonuclear leucocytes, macrophages and mast cells. These cells contain destructive enzymes within lysosomes, normally used to degrade phagocytosed material, and are capable of damaging tissue if released. Besides the response to lymphokine from immune cell, inflammatory cells can directly respond to periodontal pathogen and their products leading to a production of cytokines and enzymes affecting other cells and tissue initiating tissue damage.

While inflammatory cells and immune cells seem to play important roles in the pathogenesis of periodontitis, equally, fibroblasts, gingival and periodontal fibroblasts, are major constituents participating in the synthesis and degradation of tissue matrix component in responding to the bacteria cells and their products, and also taking important parts in disease progressing.

Gingival and PDL fibroblasts demonstrate several different characteristics. PDL fibroblasts express high levels of alkaline phosphatase (AP), transforming growth factor β1, osteocalcin and cyclic adenosine monophosphate than do gingival fibroblasts (Arceo et al., 1991; Ogata et al., 1995; Kuru et al., 1998). A study using flow cytometry revealed that gingival fibroblasts are smaller and less granular compared with the PDL cells. Regarding the expression of some extracellular matrix (ECM) molecules, PDL cells were found to express more fibronectin and collagen type I than gingival fibroblast (Kuru et al., 1998). Moreover, PDL cells also have other phenotypic features as osteoblast-like cells for example the expression of high level of osteocalcin and the formation of calcium phosphate nodules (Arceo et al., 1991). Recently, study using DNA microarray analysis between PDL cells and gingival fibroblasts shown those 163 genes were differentially expressed by at least three-fold. In PDL cells, genes encoding transmembrane proteins and cytoskeleton-related proteins tended to be up-regulated, while in gingival fibroblasts, genes encoding cell-cycle regulation protein and metabolic-related proteins tended to be up-regulated (Han and Amar, 2002). Besides distinct response to growth factors during wound healing processes (Mumford et al., 2001) as well as unique differences with respect to proliferation and wound fill (Oates et al., 2001) were observed between these cells. These evidences indicated that PDL and gingival fibroblasts might play different roles in the periodontal tissue.

Numerous studies have been taken on gingival fibroblast in various aspects whereas fewer studies on PDL fibroblast have been reported. Interestingly, PDL fibroblasts, although seem to be a smaller group of population in periodontal tissue located in the periodontal ligament space, play important roles not only in formation

and maintenance of periodontal ligament but also in the repair, remodeling and regeneration of the adjacent alveolar bone and cementum (Hou and Yaeger, 1993; Strutz et al., 1995; Nishimura and Terranova, 1996; Wise et al., 1997; Kuru et al., 1998; Lackler et al., 2000). Thus, to observe the different aspects in cellular responding in periodontitis this study was taken on PDL fibroblasts.

As mentioned above, numerous studies have been emphasized on periodontal tissue destruction through the induction of pro-inflammatory cytokines by gingival and periodontal ligament fibroblasts (Genco, 1992; Roberts et al., 1997; Mogi et al., 1999). Nevertheless, the ultimate end result of the progressing disease is the destruction of gingival connective tissue, periodontal ligament and alveolar bone which relies on the function of proteases and osteoclasts.

It has been widely accepted that enzymes in MMP family play important roles in pathogenesis of periodontal disease. Previously, some of collagenolytic MMPs, Collagenases (MMP-1,-8) and stromelysin-1 (MMP-3) (Birkedal-Hansen, 1993; Ingman et al., 1994a; Ingman et al., 1994b, c; Aiba et al., 1996; Kubota et al., 1996; Nomura et al., 1998) seem to be the prominent group in this process, because of their capability on collagen degradation especially type I collagen (MMP-1,-8). To this point, MMP-2 or gelatinase A increasingly shows its marked role in periodontal disease (Ingman et al., 1994a; Makela et al., 1994; Korostoff et al., 2000b; Dahan et al., 2001; Komatsu et al., 2001; Ejeil et al., 2003). For the reason that MMP-2 is able to degrade several kinds of extracellular matrix including collagen type I (Creemers et al., 1998), and has the tight and possesses unique regulation on activation mechanism.

Osteoclast is the only cell that is responsible for bone resorption. Multistage of osteoclast differentiation and function are regulated by several molecules namely

RANKL, RANK, OPG and M-CSF. Even though some studies report the direct activation of osteoclast by the LPS (Jiang et al., 2002; Suda et al., 2002; Jiang et al., 2003), the role of RANKL and OPG is still important. Moreover, recent reports indicated the role of RANKL and OPG in periodontitis (Teng et al., 2000; Crotti et al., 2003; Liu et al., 2003; Mogi et al., 2004).

The results from the present study emphasize the role of PDL cells on MMP-2 activation and RANKL-OPG expression which are partly in pathogenesis of periodontal disease.

MMP-2 activation in periodontal disease

As mentioned previously in this study, the MMP-2 plays roles in periodontal disease. MMP-2 is constitutively produced by PDL cells as a latent form and requires activation process to generate the active form to function. To prove MMP-2 activation mechanism in periodontal disease model may be essential for understanding pathogenesis of periodontal disease.

The results from the present study showed the increase in MMP-2 activation both from *A. actinomycetemcomitans* supernatant and LPS stimulation. However, distinct MMP-2 activation mechanisms resulting from *A. actinomycetemcomitans* supernatant and LPS have been observed. Effect of *A. actinomycetemcomitans* supernatant appears to act on TIMP-2 molecule by decreasing the TIMP-2 protein level in the conditioned media. Additionally, Phenanthroline, the MMP inhibitor, could abolish this effect, which showed that there was/were MMP(s) involved in this *A. actinomycetemcomitans* supernatant- induced MMP-2 activation mechanism. The possible MMP molecule involves in this system might be MT1-MMP presented on

PDL cell surface. These results are in accordance with the MMP-2 activation mechanism proposed by Strongin et al (1995). This mechanism involves the balance of two types of molecules, MT1-MMP and TIMP-2. The MMP-2 activation could occur only when the ratio of these molecules is proper. Up to now, the exact ratio of these molecules has not been elucidated.

In a different way, *A. actinomycetemcomitans* LPS increase MMP-2 activation without affecting MT1-MMP and TIMP-2 level. Moreover, serine protease inhibitor completely and NF-kB inhibitor partially inhibited this effect while phenanthroline, the MMP inhibitor could not. These indicated that *A. actinomycetemcomitans* LPS might use the novel pathway to activate MMP-2 which might be serine protease-dependent pathway and partially through NF-kB pathway. Further study to find out the precise mechanism in which *A. actinomycetemcomitans* LPS involve in MMP-2 activation should be investigated

Taken together these evidences showed that *A. actinomycetemcomitans* could employ multiple virulence including secreted products and LPS to cause MMP-2 activation by both the well-known mechanism using MT1-MMP and TIMP-2 and the novel mechanism, serine protease-dependent pathway. These supported the role of *A. actinomycetemcomitans* in inducing host response and the role of MMP-2 in part of soft tissue degradation.

A. actinomycetemcomitans LPS enhancing osteoclastogenesis by up-regulation of RANKL

Although there are several factors responsible for the differentiation and activation of osteoclasts, the important one is RANKL. Osteoblastic lineage cells and

activated T cells are two main cell types that express RANKL. In addition stromal cells including PDL cells also express RANKL. Evidences that support the role of RANKL in PDL cells were shown. PDL cells derived from resorbing deciduous teeth express RANKL but decrease OPG expression (Hasegawa et al., 2002; Fukushima et al., 2003). PDL cells derived from permanent teeth also express both RANKL and OPG, however, the balance of these two molecules is existed to maintain the integrity of PDL in the healthy tissue (Hasegawa et al., 2002). Imbalance of RANKL and OPG which believed to cause bone resorption were observed in PDL cells under stress (Kanzaki et al., 2002; Tsuji et al., 2004) and also in periodontal disease (Mogi et al., 2004).

The present study showed the effect of *A. actinomycetemcomitans* LPS, the potent destructive mediator in periodontal disease, on disturbing the balance of RANKL and OPG. The results revealed that *A. actinomycetemcomitans* LPS could up-regulation RANKL in PDL cells while did not have any effect on OPG level. Since serine protease inhibitor could abolish this effect in the same way as the effect on MMP-2 activation, this indicated that the up-regulation of RANKL by *A. actinomycetemcomitans* LPS could occur via serine protease-dependent pathway. These support the roles of *A. actinomycetemcomitans* LPS on enhancing alveolar bone resorption in periodontal disease progression.

LPS receptors

Although this study could not clarify the molecule(s) that act as LPS receptor on PDL cells, using anti-LBP antibody could not affect the up-regulation of RANKL

indicated that PDL cells did not respond to *A. actinomycetemcomitans* LPS via the most common LPS responsive pathway, LBP-CD14 pathway.

However, up to now, the receptor that binds with *A. actinomycetemcomitans* LPS on PDL cells remains unknown. There are at least two candidate molecules for this role. Firstly, CD14, the most common LPS receptor on mature myeloid cells, macrophages (Ziegler-Heitbrock and Ulevitch, 1993) and gingival fibroblasts (Watanabe et al., 1996; Hatakeyama et al., 2003; Wang et al., 2003; Mochizuki et al., 2004).

Recently, Mochizuki et al, (Mochizuki et al., 2004) revealed that gingival fibroblast responded to *A.actinomycetemcomitans* LPS via CD14-TLR4 pathway. In addition, study on PDL cells also found that anti-CD14 and anti-TLR4 could abolish the induced IL-8 production by LPS (Hatakeyama et al., 2003). Since CD14 is capable of binding directly to LPS without helping by LBP, CD14 is still the possible LPS receptor for this present study. Moreover, sCD14 was also found to mediate the LPS signal in many cells such as gingival epithelial cells (Uehara et al., 2001). sCD14 might be a molecule that facilitates the binding of *A. actinomycetemcomitans* LPS to unknown receptor on PDL cell surface.

Secondly, CD18, the leukocyte integrin also found to bind LPS (Wright and Jong, 1986) and induce cellular activation (Ingalls and Golenbock, 1995; Ingalls et al., 1998)

However there are still the possibility that MMP-2 activation and RANKL up-regulation by *A. actinomycetemcomitans* LPS might be mediated by other LPS receptor than CD14 or CD18. Further study to clarify this point should be elusive.

Study model

The present study is the in vitro experiment using PDL cells representing cells in periodontium that responding to periodontal pathogen infection. *A. actinomycetemcomitans* has been chosen as the etiologic agent initiating periodontal disease using its supernatant and LPS. The outcomes of the tissue destruction were examined on MMP-2 activation regarding to soft tissue degradation, and RANKL-OPG expression as regards to bone resorption. Additionally, the mechanism of this stimulation and the signal transduction following the stimulation were investigated.

Concluding Remarks

In conclusion, *A. actinomycetemcomitans*, one of the periodontal pathogen has various virulence factors both secreted form as soluble molecules in the supernatant and cell membrane component like LPS. These virulence factors could cause the periodontal tissue destruction by several mechanisms. In part of soft tissue destruction, MMP-2, one of the outstanding extracellular matrix degrading enzymes secreted by PDL cells, has been activated by both *A. actinomycetemcomitans* supernatant and LPS in PDL cells. Regarding hard tissue destruction, the ratio of RANKL and OPG, the important protein involved in osteoclastogenesis, was increased by *A. actinomycetemcomitans* LPS in PDL cells

Future studies

Outside the scope of this study, there have been some points of interest needed to be clarified

1. *A. actinomycetemcomitans* supernatant contains a number of effective molecules causing cell activation, therefore, to identify molecule(s) that cause MMP-2 activation is essential for investigate further on its/their molecular events underlying.
2. Since PDL cells are capable of secreting various kind of cytokine, to clarify whether these cytokines involve in this study model must be examined.
3. Several molecules have been identified as LPS receptors. Among them, CD14 is the most well known LPS receptor on LPS responsive cells. However, the existence of CD14 on PDL cells is still controversial. Thus, investigation of the LPS receptor and the signal transduction upon the stimulation in PDL cells is required.
4. Clarification of MMP-2 activation mechanism caused by LPS activation.
5. Although the present study shown the increase of RANKL expression with no change of OPG expression, the question still remains that whether this RANKL level is enough for inducing osteoclastogenesis. Therefore, further examination on the function of RANKL in PDL cells in inducing osteoclast differentiation and function is needed.
6. In osteoclastogenesis, besides the signal from the binding of RANKL and RANK, the signal from M-CSF is also important. Thus, elucidation of the involvement of M-CSF expression in this model might draw the better picture for the role of *A. actinomycetemcomitans* LPS on PDL cells in enhancing osteoclastogenesis.

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APPENDICES

APPENDIX A

Oral Diseases (2004) 10, 383–388

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Oral Biology

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

S Tiranathanagul, K Pattamapun, T Yongchaitrakul, P Pavasant

Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

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.
Oral Diseases (2004) **10**, 383–388

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

Correspondence: Prasit Pavasant, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Henri Dunant Road, Pathumwan, Bangkok 10330, Thailand. Tel: (662) 218 8885, Fax: (662) 218 8870, E-mail: prasit.pav@chula.ac.th
Received 28 August 2003; revised 31 December 2003 & 17 March 2004; accepted 19 April 2004

(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*.

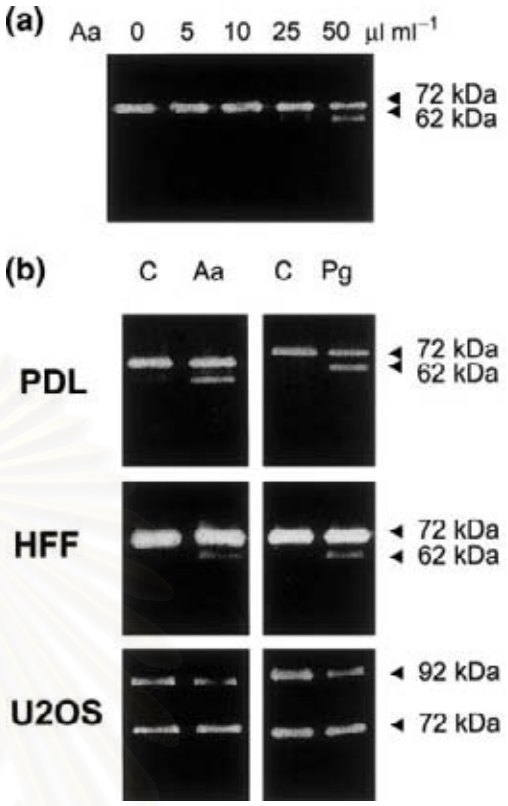

Figure 1 (**a**) HPDL cells were treated with 0, 5, 10, 25 and 50 μl ml^{-1} of supernatant for 48 h and the activation of MMP-2 was analyzed by gelatin zymography. The activation could be detected clearly when treated with 50 μl ml^{-1}. (**b**) For the gelatin zymography analysis, HPDL cells, human 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.

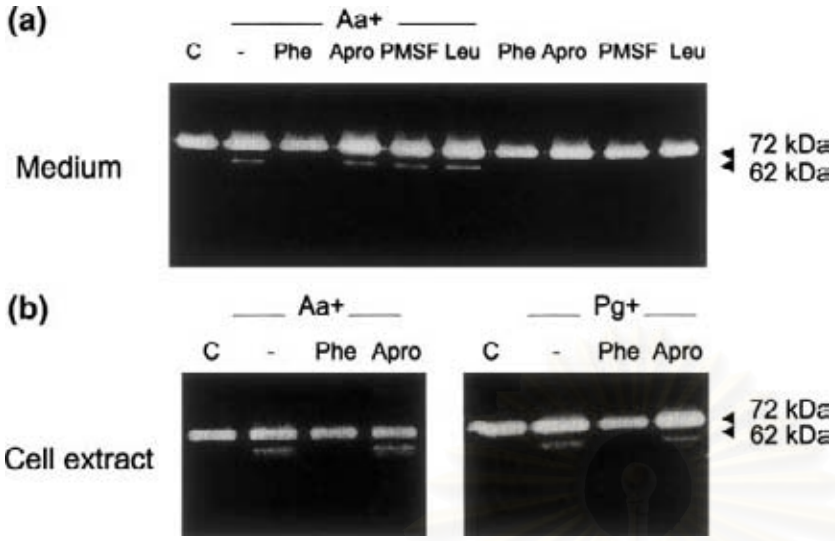

Figure 2 (**a**) Gelatin zymography showed the inhibitory effect of protease inhibitors. HPDL cells were treated with 50 μl ml^{-1} of *A. actinomycetemcomitans* (Aa) supernatant in the presence or absence of protease inhibitors; phenanthroline (Phe), aprotinin (Apro), PMSF and leupeptin (Leu). The figure 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

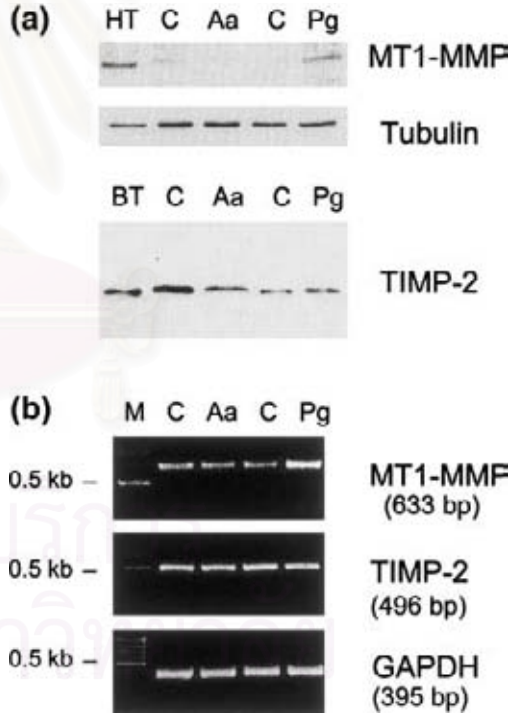

Figure 3 (**a**) The Western blot analysis of MT1-MMP and TIMP-2 after treatment with either *A. actinomycetemcomitans* (Aa) or *P. gingivalis* (Pg) supernatant. The results show that the Pg supernatant could up-regulate MT1-MMP in HPDL cells while the Aa supernatant down-regulated TIMP-2 when compared 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

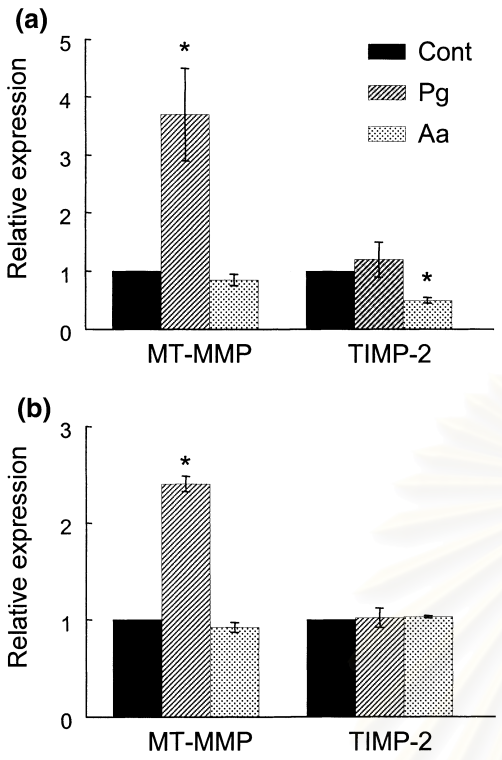

Figure 4 The histograms summarized the change of MT1-MMP and TIMP-2 from three different experiments. (**a**) The protein level, (**b**) the mRNA level of MT1-MMP and TIMP-2. The graph marked with asterisk denotes any significant differences when compared with its control (one-way ANOVA, Scheffe's test, $P < 0.05$). *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.

Acknowledgements

The authors thank Professor Erik Thompson for providing U2OS and HDF. This work is supported by the Thailand Research Fund through the Royal Golden Jubilee PhD Program (grant no. PHD/0024/2546 and RDG3/18/2543) and the Ratchadaphisek Somphot Endowment support for Research Unit, Chulalongkorn University.

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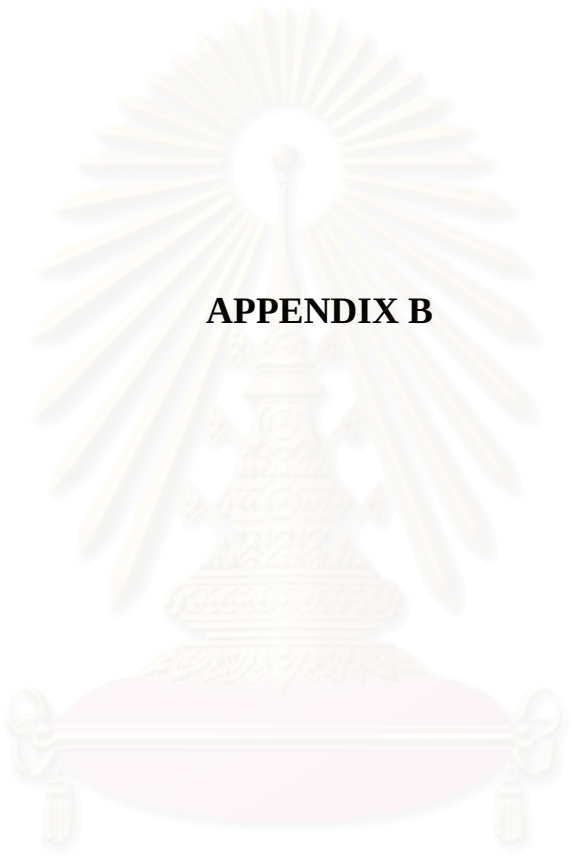
APPENDIX B



J Periodontol • December 2004

Actinobacillus actinomycetemcomitans Lipopolysaccharide Activates Matrix Metalloproteinase-2 and Increases Receptor Activator of Nuclear Factor-κB Ligand Expression in Human Periodontal Ligament Cells

Siriluck Tiranathanagul,* Tussanee Yongchaitrakul,* Kassara Pattamapun,* and Prasit Pavasant*

Background: The lipopolysaccharide (LPS) of *A. actinomycetemcomitans* is one of the major pathogenic factors in periodontal disease. It induces secretion of proinflammatory cytokines and is involved in alveolar bone destruction. We hypothesized that the LPS of *A. actinomycetemcomitans* could affect the activation of matrix metalloproteinase (MMP)-2 and the expression of receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin in human periodontal ligament (HPDL) cells leading to the destruction of periodontium.

Methods: HPDL cells were cultured in serum-free medium with or without the LPS of *A. actinomycetemcomitans* for 36 hours. The activation of MMP-2 was analyzed by zymography. Changes of the expression of RANKL and osteoprotegerin (OPG) were examined by reverse transcription-polymerase chain reaction and supported by Western blot analysis.

Results: The activation of MMP-2 could be induced by the LPS of *A. actinomycetemcomitans* in HPDL cells and could be inhibited by a serine protease inhibitor. This result suggested that the LPS might activate MMP-2 through a serine protease-dependent pathway. This activation was also blocked by NF-κB inhibitor, which indicated the involvement of NF-κB. The upregulation of RANKL but not OPG by the LPS was found in both transcription and translation and could be reduced by indomethacin. In addition, serine protease inhibitor also inhibited the upregulation of RANKL, suggesting the activity of serine protease.

Conclusions: The effect of the LPS of *A. actinomycetemcomitans* on HPDL cells is serum-independent and the induction of the activation of MMP-2 and the expression of RANKL are serine protease-dependent pathways. The results suggest the role of HPDL cells in the pathogenesis of periodontitis. *J Periodontol 2004;75:1647-1654.*

KEY WORDS

***Actinobacillus actinomycetemcomitans*; cells, periodontal; lipoprotein polysaccharide; metalloproteinases; receptor activator, nuclear factor kappa B.**

* Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.

Certain species of Gram-negative bacteria harbored in periodontal pockets play a major role in the pathogenesis of periodontitis.[1,2] Among these bacteria, *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) has been implicated as an etiological agent in juvenile and adult periodontits [1,3] *A. actinomycetemcomitans* produces a multitude of products that participate in the process of inflammation and tissue destruction. Among these products, the LPS of *A. actinomycetemcomitans* has been considered to be involved in the pathogenesis of alveolar bone loss and connective tissue degradation in periodontal disease.[4]

Lipopolysaccharide is known to induce bone resorption by enhancing osteoclastogenesis.[5] Although it is still not clear whether LPS could directly target osteoclast precursors, studies provided evidence that LPS indirectly influenced the osteoclast formation through osteoblasts or bone marrow stromal cells.[4,6] In addition, LPS is also known to induce the secretion of several inflammatory cytokines.[7,8] Increasing these cytokines causes the imbalance of proteolytic enzymes which eventually leads to connective tissue and bone destruction.[9,10]

A family of proteolytic enzymes, namely matrix metalloproteinases (MMPs), has been considered to have a role in connective tissue degradation in both physiological and pathological situations.[11,12] A number of MMPs have been detected in the crevicular fluid of patients with periodontitis and are believed to play an important role in periodontal tissue degradation.[11] These MMPs include the interstitial collagenases such as MMP-1, MMP-8, stromelysin (MMP-3), and MMP-9. Matrix metalloproteinase-2, a 72-kDa gelatinase, was shown to be involved in the pathogenesis of periodontal disease.[13,14] The amount of both latent and active MMP-2 was found to increase in the periodontal tissue of patients with periodontitis. Matrix metalloproteinase-2 is an enzyme that is secreted mainly from fibroblasts, including human gingival fibroblasts (HGF) and human periodontal ligament (HPDL) cells.[15,16] The enzyme is secreted in latent form and requires activation for proper function. In our previous study, we found that the supernatant from *Porphyromonas gingivalis (P. gingivalis)*, another etiological bacterium in periodontitis, could induce the activation of MMP-2 by increasing the expression of membrane-type matrix metalloproteinase 1 (MT1-MMP) in HPDL cells.[15] These results suggested the role of MMP-2 and HPDL cells in the process of connective tissue degradation in periodontal disease.

HPDL cells may also play a role in osteoclastogenesis through the expression of receptor activator of NF-κB ligand (RANKL) on their cell surfaces.[17-19] It is widely accepted that formation of osteoclasts requires the interaction between receptor activator of NF-κB (RANK), which is expressed on the surface of osteoclasts and its ligand, RANKL, in the presence of macrophage colony-stimulating factor (M-CSF).[20] RANKL is found on the surface of many cell types, including osteoblasts and bone marrow stromal cells. Expression of RANKL in HPDL cells leads to a hypothesis that they play a role in osteoclast formation. In addition, HPDL cells secrete a certain amount of osteoprotegerin (OPG), a decoy tumor necrosis factor (TNF) receptor, which is able to bind to RANKL.[17] An interaction between OPG and RANKL will inhibit the formation of osteoclasts. Hence, a balance of RANKL and OPG in HPDL cells may be associated with the homeostasis of the periodontium.

The purpose of this study was to examine the effect of *A. actinomycetemcomitans* LPS on HPDL cells. The study focused on the ability of LPS from *A. actinomycetemcomitans* to activate MMP-2 and subsequently alter RANKL and OPG, which are factors involved in connective tissue degradation and bone resorption.

MATERIALS AND METHODS

Cell Culture

Human PDL 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 reasons as previously described.[15] All patients gave informed consent. Briefly, teeth were rinsed with sterile phosphate buffered saline several times, and the PDLs were scraped out from the middle third of the root. The explants were harvested on a 60 mm culture dish† and grown in Dulbecco's modified Eagle medium‡ (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine,‡ 100 units/ml penicillin,‡ 100 µg/ml streptomycin,‡ and 5 µg/ml amphotericin B‡ at 37°C in humidified atmosphere of 95% air, 5% CO_2. Cells from the third to the fifth passage were used.

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

Preparation of Lipopolysaccharide

Actinobacillus actinomycetemcomitans (ATCC 43718) was cultivated in brain heart infusion broth§ (BHI) at 37°C, 5% CO_2. The LPS was prepared by water-phenol extraction as described by Wesphal and Jann.[21] The amount of LPS used in the experiment was determined by dry weight.

Activation of HPDL Cells With LPS

Human PDL cells were seeded in six-well plates† at a density of 25,000 cells/cm^2 and were allowed to attach for 16 hours. Cell activity was stopped with serum-free medium containing 0.02% lactalbumin hydrolysate‖ overnight. *A. actinomycetemcomitans* LPS was added and incubated for another 48 hours. All treatments were conducted in a serum-free condition. After 36 hours, the medium was collected and kept at –20°C until analysis.

In the inhibitory experiment, cells were treated with inhibitors for 30 minutes prior to the treatment of bacterial LPS. The inhibitors used in the experiment included 10 µM phenanthroline (phen),‖ 5 ng/ml (0.25 TIU) aprotinin (apro),‖ 50 µM pyrrolidinedithiocarbamate ammonium salt (PTDC),‖ and 1 µM indomethacin. To verify the involvement of human lipopolysaccharide binding protein (LBP), the inhibitory antibody against LBP, cloned 6G3,¶ was used.

Gelatin Zymography

The MMP-2 activity was evaluated by gelatin zymography. The medium was subjected to a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.1% gelatin under a non-reducing condition. After electrophoresis, the gel was gently shaken in a renaturing buffer (0.25% Triton-x-100) at room temperature for 30 minutes to remove SDS, and then incubated in a developing buffer (0.15 M NaCl/10 mM $CaCl_2$/50 mM

† Nunc, Naperville, IL.
‡ Gibco BRL, Carlsbad, CA.
§ Difco, Sparks, MD.
‖ Sigma Chemical Co., St. Louis, MO.
¶ HyCult Biotechnology b.v., Uden, The Netherlands.

Tris-HCL pH 7.5/0.1% Brij35) at 37°C for 20 hours. The gel was stained with 2.5% Coomassie brilliant blue in 30% methanol and 10% acetic acid. Latent and active MMP-2 can be detected as clear bands at the positions 72 kDa and 62 kDa, respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were treated with the LPS of *A. actinomycetemcomitans* as described above for 36 hours. Total cellular RNA was extracted with trizol[‡] according to manufacturer's instructions. One µg of each RNA sample was converted to cDNA by a reverse transcription using an AMV (avian myeloblastosis virus) reverse transcriptase[#] for 90 minutes at 42°C. Subsequent to the reverse transcription, a PCR was performed. The primers were prepared;[**] the oligonucleotide sequences of the primers are

MT1 MMP	forward	5′ CATCGCTGCCATGC AGAAGT 3′
	reverse	5′ GTCATATCGGGCAG CAC 3′
TIMP-2	forward	5′ GGAAGTGACTCTG GAAACGACATT 3′
	reverse	5′ CTCGATGTCGAGA AACTCCTGCTTG 3′
RANKL	forward	5′ CCAGCATCAAAATC CCAAGT3′
	reverse	5′ CCCCTTCAGATGAT CCTTC 3′
OPG	forward	5′ TGCAGTACGTCAA GCAGGAG 3′
	reverse	5′ TGACCTCTGTGAA AACAGC 3′
COX-2	forward	5′ TTCAAATGAGATTG TGGGAAAATTGCT 3′
	reverse	5′ AGATCATCTCTGCC TGAGTATCTT 3′
GAPDH	forward	5′ TGAAGGTCGAGTC AACGGAT 3′
	reverse	5′ TCACACCCATGACG AACATGG 3′

The PCR was performed using Taq polymerase with a PCR volume of 25 µl. The mixtures contained 25 pmol of primers and 1 µl of RT product. The PCR working conditions were set at a denaturation for 1 minute at 94°C, primer annealing for 1 minute at 60°C, and chain elongation for 1.45 minutes at 72°C on a DNA thermal cycler.[††] The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining.

Western Blot Analysis

RANKL and tubulin were analyzed from cell extracts using radioimmunoprecipitation buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing cocktail protease inhibitors.[‖] The amount of protein was determined by protein assay.[‡‡] 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 membrane was stained with antibody for RANKL (AB1862[§§]) or anti-tubulin antibody (a gift from Professor Erik Thompson, St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia). 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 minutes, followed by another 30 minutes staining with peroxidase-conjugated streptavidin.[‖‖] The membrane was coated with chemiluminescence reagent[‡‡] and the signals were captured.

RESULTS

Figure 1A demonstrates the effect of the LPS prepared from *A. actinomycetemcomitans* on the activation of MMP-2 in HPDL cells. The activation of MMP-2 could be identified by the presence of a 62-kDa band in the zymography. Lipopolysaccharide of *A. actinomycetemcomitans* activated MMP-2 in a dose-dependent manner, starting from 1 µg/ml of the LPS. However, no changes in the enzyme level could be observed when analyzed with a densitometer. The experiment was performed in triplicate using HPDL cells from three donors.

Human PDL cells were activated with 2 µg/ml of the LPS of *A. actinomycetemcomitans* in the presence of protease inhibitors, aprotinin, a serine protease inhibitor, and phenanthroline, an MMP inhibitor. The result (Fig. 1B) showed the disappearance of a 62-kDa band in lane 3 (+LPS, +apro) as compared to lane 2 (+LPS), and indicated that aprotinin could inhibit the effect of the LPS on the activation of MMP-2. On the contrary, no changes of the activation could be observed in the presence of phenanthroline (compare lane 2 with 4). In addition, pyrrolidinedithiocarbamate (PTDC), an NF-κB inhibitor, could also reverse the effect of the LPS on the activation (lane 5), suggesting the involvement of NF-κB in the mechanism of activation by the LPS of *A. actinomycetemcomitans*.

Figure 2 shows RT-PCR analysis of the expression of MT1-MMP and TIMP-2 after treatment with the LPS of *A. actinomycetemcomitans* for 36 hours. No change of the expression of MT1-MMP and TIMP-2 was found when compared to the control. The results corresponded with those from the inhibitory experiment, which demonstrated that phenanthroline could not inhibit the activation of MMP-2 induced by the LPS of

Promega, Madison, WI.
** Genset Biotech, Singapore.
†† ThermoHybaid, Ashford, U.K.
‡‡ Pierce, Rockford, IL.
§§ Chemicon, Temecula, CA.
‖‖ Zymed, South San Francisco, CA.

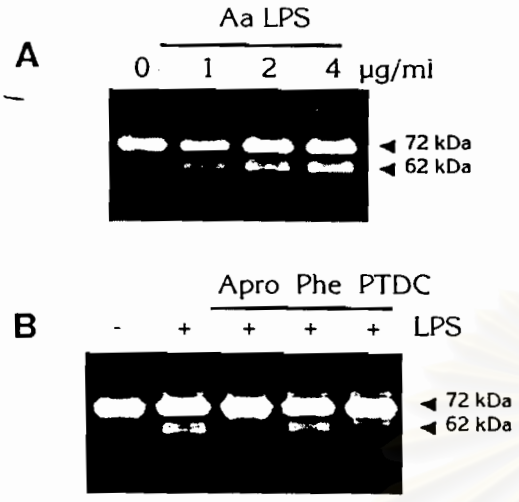


Figure 1.
Gelatin zymography demonstrates the effect of LPS from A. actinomycetemcomitans on the activation of MMP-2 **(A)**. *Addition of the LPS to the HPDL cell culture induces the activation of MMP-2 in a dose-dependent manner.* **B)** *Inhibitory effect of Apro and PTDC on the activation of MMP-2 induced by +LPS, while Phe does not show the inhibitory effect on the activation. Arrow heads indicate the positions of latent (72 kDa) and active (62 kDa) MMP-2.*

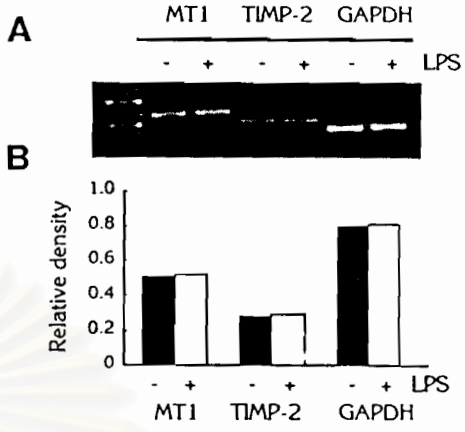


Figure 2.
An analysis of MT1-MMP and TIMP-2 by RT-PCR. **A)** *Level of MT1-MMP (MT1) and the expression of TIMP-2 in HPDL cells after treatment with 4 μg/ml of A. actinomycetemcomitans LPS (+LPS). No change in levels of MT1-MMP or TIMP-2 is observed compared to GAPDH.* **B)** *Relative density of the PCR product in A.*

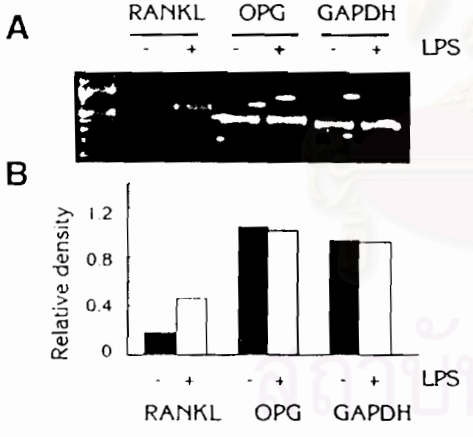


Figure 3.
An analysis of RANKL and OPG by RT-PCR. **A)** *Expression of RANKL and OPG in HPDL cells after treatment with 4 μg/ml of the LPS (+LPS). The relative density of the PCR product is shown in* **B**.

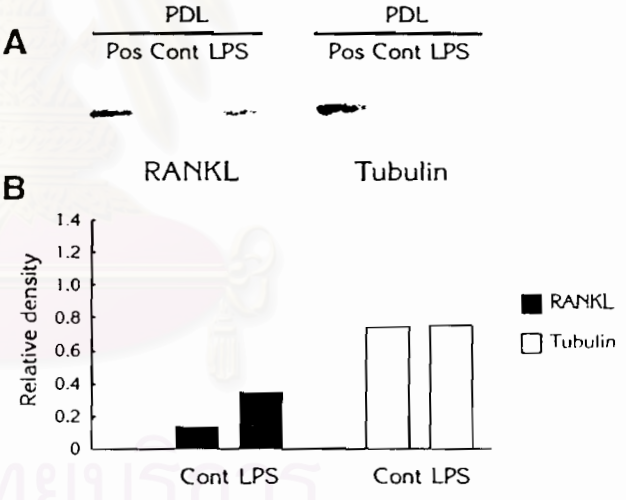


Figure 4.
Western blot analysis of the expression of RANKL on HPDL cells after treatment with A. actinomycetemcomitans LPS **(A)**. *The protein extracted from SaOS-2 is used as positive control (Pos). The relative density of RANKL is shown in* **B**. *Tubulin is used as internal control.*

A. actinomycetemcomitans and the mechanism of the activation was not an MMP-dependent pathway.

The expression of RANKL and OPG after treatment with the LPS of *A. actinomycetemcomitans* is shown in Figure 3. An increase of RANKL expression was observed while the level of OPG expression remained the same. We further examined the effect of the LPS on the expression of RANKL using Western blot analysis (Fig. 4). A similar result was obtained since the amount of RANKL increased as compared to the control.

Addition of aprotinin to the culture medium could prevent the upregulation of RANKL induced by the LPS of *A. actinomycetemcomitans* (Fig. 5). The results also revealed that the effect of the LPS on RANKL was reduced by indomethacin, a non-specific COX inhibitor. The inhibitory effect of indomethacin suggested

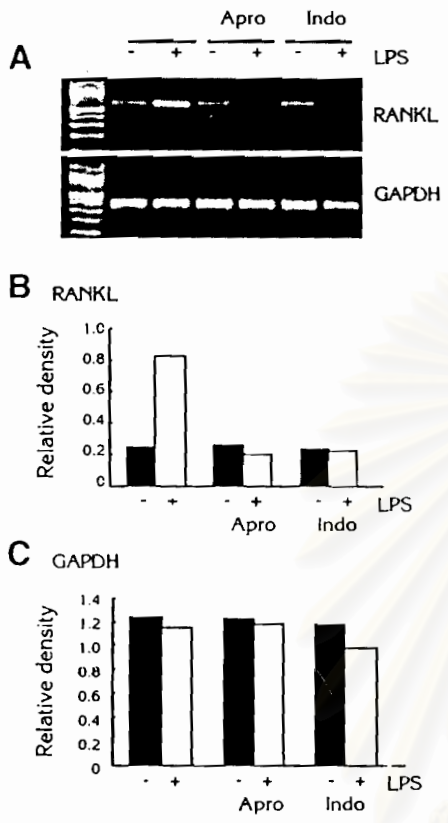


Figure 5.
RT-PCR analysis shows the effect of Apro and Indo on RANKL expression after treatment with A. actinomycetemcomitans LPS. Both aprotinin and indomethacin inhibit the inductive effect of LPS on RANKL expression ***(A)****.* ***B*** *and* ***C*** *show the relative density of PCR products of RANKL and GAPDH in A, respectively.*

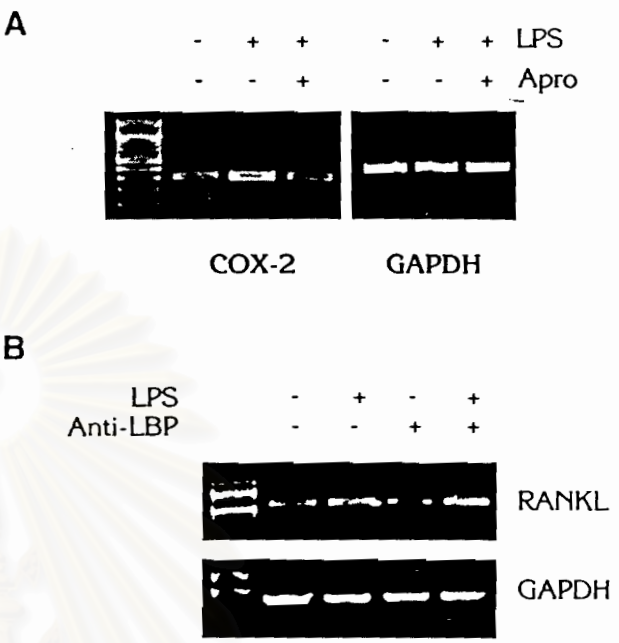


Figure 6.
A) *RT-PCR analysis indicates the inhibitory effect of Apro on the expression of COX-2 induced by the LPS.* ***B)*** *RT-PCR analysis of RANKL expression in HPDL cells induced by the LPS in the presence or absence of inhibitory antibody against human LPS-binding protein (anti-LBP).*

the COX pathway in the mechanism of RANKL induction.

Figure 6A revealed the upregulation of COX-2 in HPDL cells after treatment with the LPS. It is interesting to note that application of aprotinin could inhibit an increase of COX-2 that was induced by the LPS of *A. actinomycetemcomitans*. The results suggested that the initial signal of LPS-induced RANKL in HPDL cells might be an activity of serine protease.

To determine if LPS-binding protein (LBP) remained in our serum-free system, we added the neutralized antibody to LBP to the medium before treatment with the LPS (Fig. 6B). Addition of the anti-LBP did not inhibit the effect of the LPS on RANKL, suggesting that the response of HPDL cells to the LPS might not involve the LBP-CD14 signaling pathway.

DISCUSSION

We found that HPDL cells could respond directly to the LPS of *A. actinomycetemcomitans*. In general, LPS binds to a cell surface protein, CD14, with the help of LBP, which is present in the serum. A transmembrane signaling is then initiated by the Toll-like receptor (TLR), which has been shown to link to LPS/LBP/CD14 complex.[22,23] Due to the requirement for LBP, CD14-mediated pathways are serum-dependent. However, we used a serum-free condition in this study, indicating that the response of HPDL cells might not occur through CD14. This concept is further supported by adding a neutralized antibody to LBP to the medium. The increase of RANKL was still observed in the presence of the neutralized antibody, suggesting a different mechanism other than the LBP-CD14 signaling pathway. Kim and Koh[24] reported similar results when they treated endothelial cells with LPS in a serum-free condition. The exact mechanism of the response of HPDL cells to LPS remains to be elucidated.

Lipopolysaccharides can induce MMP-2 activation in HPDL cells. Korostoff et al.[14] reported that the level of active MMP-2 increased in the periodontal tissue of patients with periodontitis. The function of MMP-2 involves the degradation of type IV collagen in the basement membrane and denatured type I collagen.[25] Lately, MMP-2 has been shown to be able to degrade native type I collagen and has been considered a major enzyme involved in the turnover of soft connective tis-

sue.[26] The increase of active MMP-2 in periodontitis suggests a role of this enzyme in the degradation of periodontium.

We have also tested the effect of LPS from *Fusobacterium nucleatum* (*F. nucleatum*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) to investigate the specificity of the LPS from *A. actinomycetemcomitans* (data not shown). The results revealed that the LPS from *F. nucleatum* and *P. aeruginosa* also activated MMP-2 and increased RANKL expression. Our results indicate that the ability of LPS to activate MMP-2 and to induce RANKL expression is not limited to certain bacterial species. This ability might be a characteristic of LPS from several species of Gram-negative bacteria. The result is in concurrence with the report from others[24] demonstrating that the LPS of *Escherichia coli* (*E. coli*) activated MMP-2.

Our previous study[15] showed that HPDL cells responded to the supernatant from *P. gingivalis* by increasing the level of active MMP-2 and MT1-MMP. MT1-MMP is a membrane-bound MMP that functions not only in degradation of the tissue but also in a process of MMP-2 activation. The upregulation of MT-MMP has been shown to correspond with the activation of MMP-2.[27,28] However, we found that phenanthroline, a zinc chelator that acts as an MMP inhibitor, could not inhibit the activation by LPS of *A. actinomycetemcomitans*, suggesting that the process of activation might be different from the mechanism described above. An inhibition by aprotinin suggested that the mechanism might occur through a serine protease-dependent pathway. Our finding is similar to the report by Takeda et al.[29] who found that LPS of *E. coli* contained an activity of serine protease to activate MMP-2 in cervical fibroblasts.

Since the structure of LPS typically consists of a hydrophobic domain or lipid-A, a core oligosaccharide, and a distal polysaccharide or O-antigen, the presence of protease activity in LPS does not correspond with its structure. However, it is possible that LPS might induce the activity of serine protease from HPDL cells, which involves the process of MMP-2 activation. Generally, activation of MMPs requires a function of serine protease such as plasmin,[30] but that of MMP-2 requires different molecules. An accepted model of MMP-2 activation is an MMP-dependent pathway. The model was proposed by Strongin et al.,[31] who described the formation of tri-molecular complex of MMP-2, MT1-MMP, and TIMP-2 as an initial step of the activation. The prodomain of MMP-2 is then cleaved by another MT1-MMP molecule to yield a 68-kDa intermediate form of MMP-2, which is further processed into a 62-kDa, active MMP-2. However, there are few reports describing the function of serine protease in MMP-2 activation; for example, the activation of MMP-2 in astrocytes from glioma by plasminogen activator or by proteinase-3, a serine protease from polymorphonuclear leukocytes.[32,33] The evidence suggests the possible action of serine protease in the activation of MMP-2.

In addition, our results revealed that PTDC, an NF-κB inhibitor, could inhibit the activation of MMP-2 induced by the LPS of *A. actinomycetemcomitans*. The NF-κB has been shown to be involved in the regulation of both serine proteases and MMPs.[24,28,34] The function of NF-κB also involves MMP-2 activation through the function of MT1-MMP.[24,28] In addition, it has been reported that LPS could induce a production of inflammatory cytokines through an NF-κB-dependent pathway,[35] which might affect the activation of MMP-2. However, the exact role of NF-κB in the activation of MMP-2 in HPDL cells induced by the LPS needs further investigation.

Apart from the function of MMP-2 activation, we also found that the LPS of *A. actinomecetemcomitans* induced the expression of RANKL in HPDL cells. RANKL is a surface protein that plays an important role in osteoclast formation. Generally, LPS has been considered to be a potent inducer of osteoclast formation. It has been shown that LPS induced RANKL expression in osteoblasts and marrow stromal cells.[5] The increase of RANKL in these cells may provide a support for osteoclastogenesis and results in the increased number of osteoclasts in vitro.

Recent reports revealed that the expression of RANKL increased in the gingival tissue of patients with periodontitis.[18,19] The increase of RANKL corresponds with that of bone resorption, which is a major characteristic of periodontal disease, suggesting the role of RANKL in the pathogenesis of periodontitis. Furthermore, the function of RANKL is not limited to osteoclastogenesis, but also involves activation and survival of osteoclasts via a binding between RANK and RANKL.[36,37] Thus, an increase of RANKL induced by the LPS in HPDL cells might be significant in the process of alveolar bone resorption in periodontitis.

Expression of several factors, such as interleukin-11, tumor necrosis factor-α, and prostaglandin E_2 (PGE_2) has been shown in the expression of RANKL in bone and marrow stromal cells.[20,38] In PDL cells, Kanzaki et al.[39] found that application of mechanical stress to PDL cells in vitro increased the expression of RANKL through PGE_2 synthesis. A similar mechanism might have occurred in this study since indomethacin exerted a blocking effect on elevation of RANKL in the LPS-treated HPDL cells.

Application of aprotinin could block the effect of the LPS of *A. actinomycetemcomitans* in RANKL induction. Since aprotinin could inhibit both MMP-2 activation and RANKL induction, it is possible that the serine protease involved in the activation of MMP-2 plays a role in the induction of RANKL. Taking into account that aprotinin could inhibit COX-2 expression induced by the LPS suggests that the action of serine protease might be

responsible for both the activation of MMP-2 and the upregulation of RANKL in HPDL cells. A mechanism of the activity of serine protease, either from the LPS itself or from the LPS-induced HPDL cells, is still unclear.

In conclusion, we report here that the LPS of *A. actinomycetemcomitans* could induce the activation of MMP-2 and the expression of RANKL in HPDL cells. The induction might be involved by the activity of serine protease. This finding supports the concept that HPDL cells can respond directly to the LPS of *A. actinomycetemcomitans* and participate in the destruction of periodontium.

ACKNOWLEDGMENT

This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Doctorate Program (grant PHD/0024/2546), Ratchadapisek Somphot Endowment and Grant Supports for Research Unit, Chulalongkorn University.

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Correspondence: Dr. Prasit Pavasant, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Henri Dunant Road, Pathumwan, Bangkok 10330, Thailand. Fax: 66-2-218-8870; e-mail: prasit.pav@chula.ac.th.

Accepted for publication March 24, 2004.

APPENDIX C

Primers used for the RT-PCR analysis

Gene	Primers	GI no.	location	Product size (bp)	Cycle number
MT1-MMP	forward, 5' CATCGCTGCCATGCAGAAGT 3' reverse, 5' GTCATCATCGGGCAGCAC 3'	**4826833**	**305 - 938**	**633**	**28**
TIMP-2	forward, 5' GGAAGTGGACTCTGGAAACGACATT 3' reverse, 5' CTCGATGTCGAGAAACTCCTGCTTG 3'	**9257247**	**461 - 956**	**496**	**24**
RANKL	forward, 5' CCAGCATCAAAATCCCAAGT 3' reverse,5' CCCCTTCAGATGATCCTTC 3'	**2612921**	**881 - 1465**	**584**	**32**
OPG	forward, 5' TGCAGTACGTCAAGCAGGAG 3' reverse, 5' TGACCTCTGTGAAAACAGC 3'	**2072184**	**382 - 934**	**552**	**26**
COX-2	forward, 5' TTC AAA TGA GAT TGT GGG AAA GCT 3' reverse, 5'AGA TCA TCT CTG CCT GAG TAT CTT 3'	**181253**	**588 - 878**	**290**	**26**
GAPDH	forward, 5' TGAAGGTCGGAGTCAACGGAT 3' reverse, 5' TCACACCCATGACGAACATGG 3'	**4503912**	**71 - 466**	**392**	**22**

VITA

Miss Siriluck Tiranathanagul was born on November 26, 1974 in Bangkok, Thailand. She received the degree of Doctor of Dental Surgery from Chulalongkorn University in 1997. After working as a lecturer in the Department of Stomatology, Faculty of Dentistry, Srinakarinwiroj University from April 1997 – June 1999, she started her graduate study for the Degree of Doctor of Philosophy in Oral Biology, Faculty of Dentistry, Chulalongkorn University. Most of the research works were conducted at the Faculty of Dentistry, Chulalongkorn University from June 2000 – July 2003, Some parts were performed at St.Vincent's Institute of Medical Research, Melbourne, Australia, in the laboratory of Associate Professor Dr. Erik W. Thompson from August 2003 – June 2004. At present she continues her work in the Department of Stomatology, Faculty of Dentistry, Srinakarinwiroj University.