จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัยฉบับสมบูรณ์

ผลของ LPS จาก *Actinobacillus actinomycetemcomitans* ต่อ MMP-2 และการเปลี่ยนแปลงของ RANKL และ OPG ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์

ทัศนีย์ ยงชัยตระกูล และ ประสิทธิ์ ภวสันต์

พฤศจิกายน 2547

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ อาจารย์และบุคลากรของภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยที่ให้ความช่วยเหลือในการเก็บเนื้อเยื่อฟันจากผู้ป่วย ขอบคุณทันตแพทย์หญิง สิริลักษณ์ ตีรณธนากุล ที่ให้ความช่วยเหลือในการเพาะเลี้ยงเซลล์ ขอบคุณคณาจารย์ภาควิชากายวิภาคศาสตร์ที่ให้การสนับสนุนทั้งในเรื่องเวลาและสถานที่ และขอบคุณคณะอนุกรรมการบริหารทุนวิจัย กองทุนรัชดาภิเษกสมโภชที่ให้การสนับสนุนเงินทุนวิจัย

ชื่อโครงการวิจัย : ผลของ LPS จาก *Actinobacillus actinomycetemcomitans* ต่อ MMP-2 และการเปลี่ยนแปลงของ RANKL และ OPG ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์

ชื่อผู้วิจัย : ทัศนีย์ ยงชัยตระกูล และ ประสิทธิ์ ภวสันต์

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บทคัดย่อ

บทนำ: LPS ของเชื้อ *A. actinomycetemcomitans* เป็นหนึ่งในปัจจัยหลักที่ก่อให้เกิดโรคปริทันต์อักเสบ โดยเหนี่ยวนำการหลั่งไซโตไคน์ที่เกี่ยวกับการอักเสบ และการทำลายกระดูกเบ้าฟัน เราได้ตั้งสมมติฐานว่า LPS ของเชื้อ *A. actinomycetemcomitans* สามารถกระตุ้นการทำงานของ MMP-2 และการแสดงออกของ RANKL และ OPG ในเซลล์ HPDL ซึ่งนำไปสู่การทำลายของเนื้อเยื่อปริทันต์

วิธีการวิจัย: เซลล์ HPDL ถูกเลี้ยงในอาหารเลี้ยงเซลล์ที่ปราศจากซีรัม พร้อมกับมีหรือไม่มี LPS ของ *A. actinomycetemcomitans* เป็นเวลา 36 ชั่วโมง วิเคราะห์การกระตุ้น MMP-2 ด้วยไซโมกราฟฟี วิเคราะห์การแสดงออกของ RANKL และ OPG ด้วย RT-PCR และตรวจสอบด้วยเวสเทอร์นอนาไลซิส

ผลการวิจัย: การกระตุ้นการทำงานของ MMP-2 ในเซลล์ HPDL สามารถถูกเหนี่ยวนำด้วย LPS ของ *A. actinomycetemcomitans* และสามารถยับยั้งได้ด้วยสารยับยั้งต่อซีรีนโปรตีเอส แสดงว่า LPS อาจจะกระตุ้น MMP-2 โดยผ่านซีรีนโปรตีเอส การกระตุ้นยังสามารถถูกยับยั้งได้ด้วยสารยับยั้งต่อ NF-kB แสดงว่าน่าจะมีส่วนเกี่ยวข้องกับ NF-kB ด้วย LPS ไม่กระตุ้นการเพิ่มขึ้นของ OPG แต่เพิ่มระดับของ RANKL ทั้งในระดับอาร์เอ็นเอและโปรตีน ซึ่งการเพิ่มขึ้นนี้สามารถถูกยับยั้งด้วยอินโดเมทาซิน นอกจากนี้ สารยับยั้งซีรีนโปรตีเอสสามารถยับยั้งการเพิ่มของ RANKL ได้ เป็นการแสดงว่าซีรีนโปรตีเอสน่าจะมีบทบาทเกี่ยวข้องกับ RANKL

บทสรุป: ผลของ LPS จากเชื้อ *A. actinomycetemcomitans* ต่อเซลล์ HPDL ไม่ขึ้นกับซีรัม การกระตุ้นการทำงานของ MMP-2 และการแสดงออกของ RANKL เกิดผ่านซีรีนโปรตีเอส ผลการวิจัยนี้แสดงถึงอีกบทบาทหนึ่งของเซลล์ HPDL ในการเกิดโรคปริทันต์อักเสบ

คำสำคัญ: *A. actinomycetemcomitans*, LPS, MMP-2, RANKL, เซลล์ HPDL

Project Title : Effect of LPS of *Actinobacillus actinomycetemcomitans* lipopolysaccharide on matrix metalloproteinase-2 and changes of RANKL and OPG in HPDL cells.

Name of the Investigators: Tussanee Yongchaitrakul and Prasit Pavasant

Date : November 2004

Abstract

Background: The LPS of *A. actinomycetemcomitans* is one of the major pathogenic factors in periodontal disease. It induces secretion of pro-inflammatory cytokines and involves in alveolar bone destruction. We hypothesized that the LPS of *A. actinomycetemcomitans* could affect the activation of MMP-2 and the expression of RANKL and OPG in 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 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. The result suggested that the LPS might activate MMP-2 through a serine protease-dependent pathway. The activation was also blocked by NF-κB inhibitor, which indicated the involvement of NF-κB. The up-regulation of RANKL but not OPG by the LPS was found in both transcription and translation and could be abolished by Indomethacin. In addition, serine protease inhibitor also inhibited the up-regulation of RANKL, suggesting the activity of serine protease.

Conclusion: 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 pathway. The results suggest the role of HPDL cells in the pathogenesis of periodontitis.

Keywords : *A. actinomycetemcomitans*, LPS, MMP-2, RANKL, PDL cells

สารบัญ

รายการภาพประกอบ

หน้า

Introduction

In periodontitis, certain species of Gram-negative bacteria harbored in periodontal pockets play a major role in the pathogenesis of the disease.[1,2] Among these bacteria, *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) has been implicated as an etiological agent in juvenile and adult periodontits.[1,3] *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.[4]

Lipopolysaccharide was known to be an inducer of bone resorption by enhancing osteoclastogenesis.[5] 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.[4,6] In addition, LPS is also known able to induce the secretion of several inflammatory cytokines.[7,8] Increasing of 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 or MMPs, has been considered to play roles 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 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.[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 or 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 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.

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 reason as previously described.[15] 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[†] and grown in Dulbecco's Modified Eagle Medium[‡] (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine[‡], 100 units/ml penicillin[‡], 100 μg/ml streptomycin[‡], and 5 μg/ml amphotericin B[‡] at 37 °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) at 37°C, 5% CO_2. The LPS was prepared by water-phenol extraction as described by Wesphal and Jann.[21] 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[†] 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[¨] overnight, the LPS of *A. actinomycetemcomitans* 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 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". To verify the involvement of human lipopolysaccharide binding protein (LBP), the inhibitory antibody against LBP, cloned 6G3[¶], 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[‡] 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 1.5 hours at 42°C. Subsequent to the reverse transcription, a polymerase-chain reaction was performed. The

primers were prepared** following the reported sequences from GenBank. The oligonucleotide sequences of the primers are

MT1 MMP	forward	5' CATCGCTGCCATGCAGAAGT 3'
	reverse	5' GTCATCATCGGGCAGCAC 3'
TIMP-2	forward	5' GGAAGTGGACTCTGGAAACGACATT 3'
	reverse	5' CTCGATGTCGAGAAACTCCTGCTTG 3'
RANKL	forward	5' CCAGCATCAAAATCCCAAGT3'
	reverse	5' CCCCTTCAGATGATCCTTC 3'
OPG	forward	5' TGCAGTACGTCAAGCAGGAG 3'
	reverse	5' TGACCTCTGTGAAAACAGC 3'
COX-2	forward	5' TTC AAA TGA GAT TGT GGG AAA ATT 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 Tag 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.†† 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.* 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.

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† Nunc, Naperville, IL

‡ Gibco BRL, Carlsbad, CA

§ Difco, Sparks, MD

" Sigma Chemical Co., St. Louis, MO

¶ HyCult biotechnology b.v., Uden, the Netherlands

Promega, Madison, WI

•• Genset Biotech, Singapore

†† ThermoHybaid, Ashford, UK

‡‡ Pierce, Rockford, IL

§§ Chemicon Temecula, CA

"" Zymed, South San Francisco, CA

Results

The result in figure 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 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 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-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 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. 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 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 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 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 (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 in LBP-CD14 signaling pathway.

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.[22,23] 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 neutralized antibody to LBP to the medium. The increase of RANKL is still observed in the presence of the neutralized antibody suggesting the different mechanism other than the LBP-CD14 signaling pathway. Kim and Koh[24] 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.[14] 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.[25] 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.[26] 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[24] demonstrating that the LPS of *Escherichia coli* (*E. coli*) caused the activation of 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-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.[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 is typically consist 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[30] 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.[31], 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.[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-kB has been shown to involve in the regulation of both serine proteases and MMPs.[24,28,34] The function of NF-kB also involves in 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-kB-dependent pathway[35], 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.[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 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.[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 IL-11, TNF-alpha and prostaglandin E2, have been shown to involve 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. 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.

References

1. Slots J, Listgarten MA. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J Clin Periodontol* 1988;15:85-93.
2. Haffajee AD, Socransky S. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 1994;5:78-111.
3. Zambon JJ, Christersson LA, Genco RJ. Diagnosis and treatment of localized juvenile periodontitis. *J Am Dent Assoc* 1986;115:295-299.
4. Ueda N, Koide M, Ohguchi M, et al. Involvement of prostaglandin E_2 and interleukin-1α in the differentiation and survival of osteoclasts induced by lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4. *J Periodont Res* 1998;33:509-516.
5. Zou W, Bar-Shavit Z. Dual modulation of osteoclast differentiation by lipopolysaccharide. *J Bone Miner Res* 2002;17:1211-1218.
6. Pelt P, Zimmermann B, Ulbrich N, Berimoulin J-P. Effects of lipopolysaccharide extracted from *Prevotella intermedia* on bone formation and on the release of osteolytic mediators by fetal mouse osteoblasts in vitro. *Arch Oral Biol* 2002;47:859-866.
7. Saglie FR, Simon K, Merrill J, Koeffler HP. Lipopolysaccharide from *Actinobacillus actinomycetemcomitans* stimulates macrophages to produce interleukin-1 and tumor necrosis factor mRNA and protein. *Oral Microbiol Immunol* 1990;5:256-262.
8. Kesavalu L, Chandrasekar B, Eberole JL. In vivo induction of proinflammatory cytokines in mouse tissue by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 2002;17:177-180.
9. Schwartz Z, Goultschin J, Dean DD, Boyan BD. Mechanisms of alveolar bone destruction in periodontitis. *Periodontol 2000* 1997;14:158-172.

10. Siwik DA, Chang DLF, Colucci WS. Interleukin-1β and tumor necrosis factor-α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res* 2000;86:1259-1265.

11. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 1993;64:474-484.

12. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;10:602-608.

13. Mäkelä M, Salo T, Uitto V-J, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 1994;73:1397-1406.

14. Korostoff JM, Wang JF, Sarment DP, Stewart JCB, Feldman RS, Billings PC. Analysis of in situ proteinase activity in chronic adult periodontitis patients: expression of activated MMP-2 and a 40 kDa serine proteinase. *J Periodontol* 2000;71:353-360.

15. Pattamapun K, Tiranathanagul S, Yongchaitrakul T, Kuwatanasuchat J, Pavasant P. Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells. *J Periodont Res* 2003;38:115-121.

16. Lapp CA, Lohse JE, Lewis JB, et al. The effects of progesterone on matrix metalloproteinases in cultured human gingival fibroblasts. *J Periodontol.* 2003;74:277-288.

17. Hasegawa T, Yoshimura Y, Kikuiri T, et al. Expression of receptor activator of NF-kappa B ligand and osteoprotegerin in culture of human periodontal ligament cells. *J Periodont Res* 2002;37:405-411.

18. Liu D, Xu JK, Figliomeni L, et al. Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction. *Int J Mol Med* 2003;11:17-21.

19. Crotti T, Smith MD, Hirsch R, Soukoulis S, Weedon H, Capone M, Ahern MJ, Haynes D. Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J Periodontal Res* 2003;38:380-387.

20. Suda T, Takahashi N, Udagawa N, Jimi E Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999;20:345-357.

21. Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Meth Carbohydr Chem* 1965;5:83-91.

22. Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. *J Biol Chem* 1995;10:5320-5325.

23. Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature* 1998;17:284-288.

24. Kim HG, Koh GY. Lipopolysaccharide activates matrix metalloproteinase-2 in endothelial cells through an NF-kB-dependent pathway. *Biochem Biophys Res Commun* 2000;269:401-405.

25. Ellerbroek SM, Stack MS. Membrane associated matrix metalloproteinases in metastasis. *BioEssays* 1999;21:940-949.

26. Creemers LB, Jansen ID, Docherty AJ, Reynolds JJ, Beertsen W, Everts V. Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol* 1998;17:35-46.

27. Sounni NE, Baramova EN, Munaut C, et al. Expression of membrane type 1 matrix metalloproteinase (MT1-MMP) in A2058 melanoma cells is associated with MMP-2 activation and increased tumor growth and vascularization. *Int J Cancer* 2002;98(1):23-28.

28. Philip S, Bulbule A, Kundu GC. Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kappa B-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. *J Biol Chem* 2001;276:44926-44935.

29. Takeda M, Imada K, Sato T, Ito A. Activation of human progelatinase A/promatrix metalloproteinase 2 by *Esterichia coli*-derived serine proteinase. *Biochem Biophys Res Commun* 2000;268:128-132.

30. Murphy G, Stanton H, Cowell S, et al. Mechanisms for pro matrix metalloproteinase activation. *APMIS*. 1999;107:38-44.

31. Strongin AY, Collier I, Bannikov G, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. *J Biol Chem* 1995;270:5331-5338.

32. Pezzato E, Dona M, Sartor L, et al. Proteinase-3 directly activates MMP-2 and degrades gelatin and Matrigel; differential inhibition by (-)epigallocatechin-3-gallate. *J Leukoc Biol* 2003;74:88-94.

33. Le DM, Besson A, Fogg DK, et al. Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. *J Neurosci* 2003;23:4034-4043.

34. Sliva D, English D, Lyons D, Lloyd FP Jr. Protein kinase C induces motility of breast cancers by upregulating secretion of urokinase-type plasminogen activator through activation of AP-1 and NF-kappaB. *Biochem Biophys Res Commun*. 2002;290:552-557.

35. Yoza BK, Hu JY, McCall CE. Protein-tyrosine kinase activation is required for lipopolysaccharide induction of interleukin 1beta and NFkappaB activation, but not NFkappaB nuclear translocation. *J Biol Chem* 1996;271:18306-18309.

36. Takami M, Woo JT, Nagai K. Osteoblastic cells induce fusion and activation of osteoclasts through a mechanism independent of macrophage-colony-stimulation factor production. *Cell tissue Res* 1999;298:327-334.

37. Udagawa N, Takahashi N, Jimi E, et al. Osteoblasts/stroaml cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony stimulation factor: receptor activator of NF-kappa B ligand. *Bone* 1999;25:517-523.

38. Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* 1998;95:3597-3602.

39. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor κB ligand up-regulation via prostaglandin E2 synthesis. *J Bone Miner Res* 2002;17:210-220.

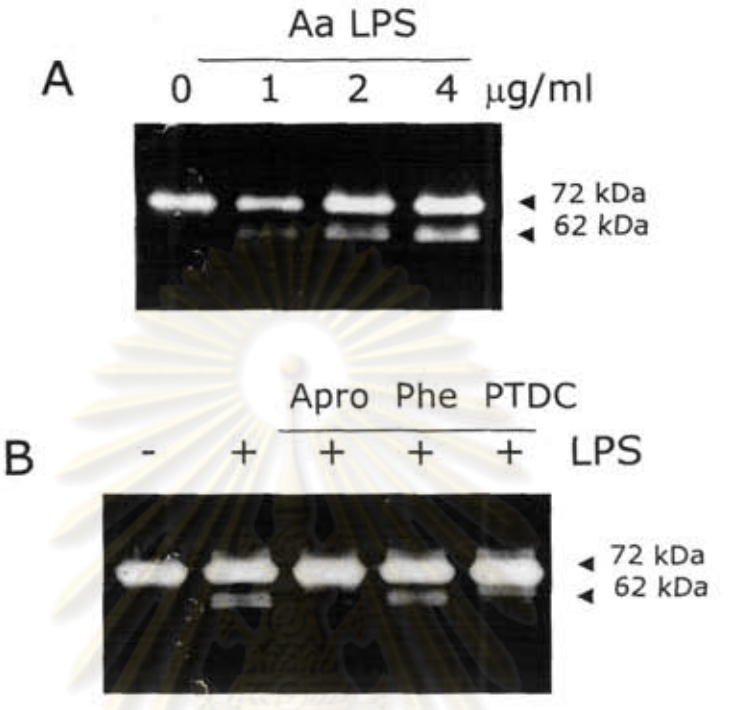

Figure 1

Figure 1 Gelatin zymography demonstrates the effect of LPS from *A. actinomycetemcomitans* on the activation of MMP-2 (Fig.1A). Addition of the LPS to the culture of HPDL cells induces the activation of MMP-2 in a dose dependent manner. Fig.1B 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.

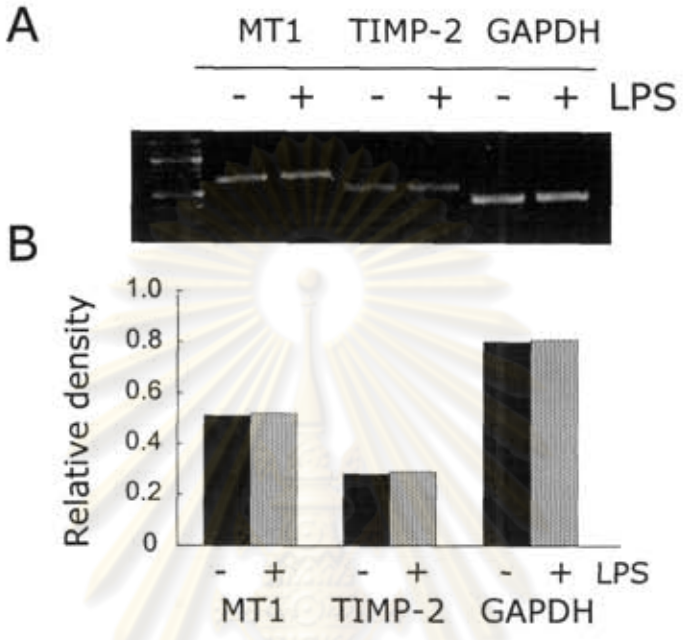

Figure 2

Figure 2 An analysis of MT1-MMP and TIMP-2 by RT-PCR. Fig.2A shows the level of MT1-MMP (MT1) and the expression of TIMP-2 in HPDL cells after treatment with 4 mg/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. Fig.2B indicates a relative density of the PCR product in Fig.1A. The figure represents one of three experiments.

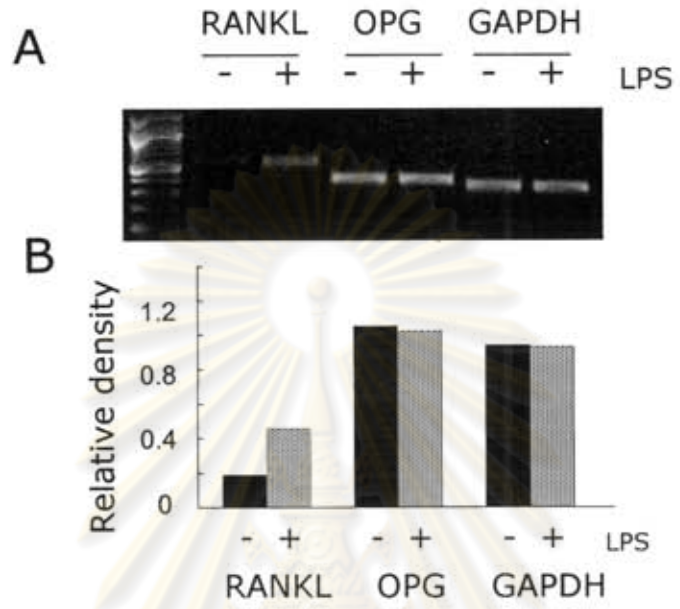

Figure 3

Figure 3 An analysis of RANKL and OPG by RT-PCR. Fig.3A reveals the expression of RANKL and OPG in HPDL cells after treatment with 4 mg/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.

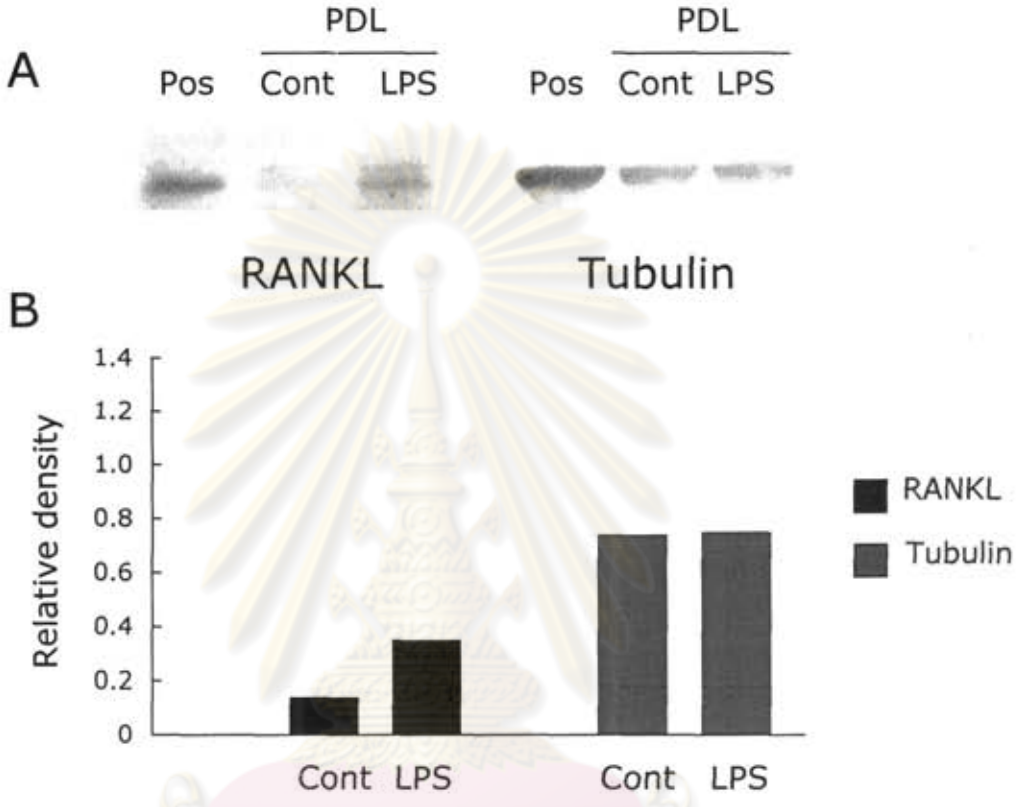

Figure 4

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

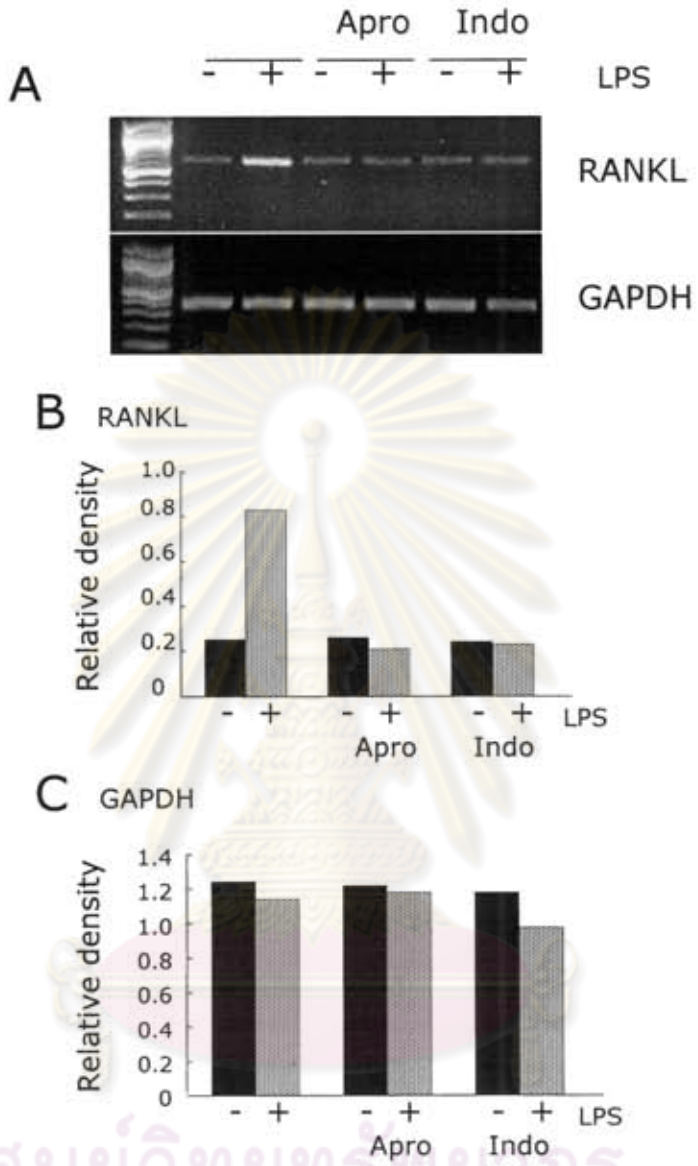

Figure 5

Figure 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 (Fig.5A). Fig.5B and C show the relative density of PCR products of RANKL and GAPDH in Fig.5A, respectively.

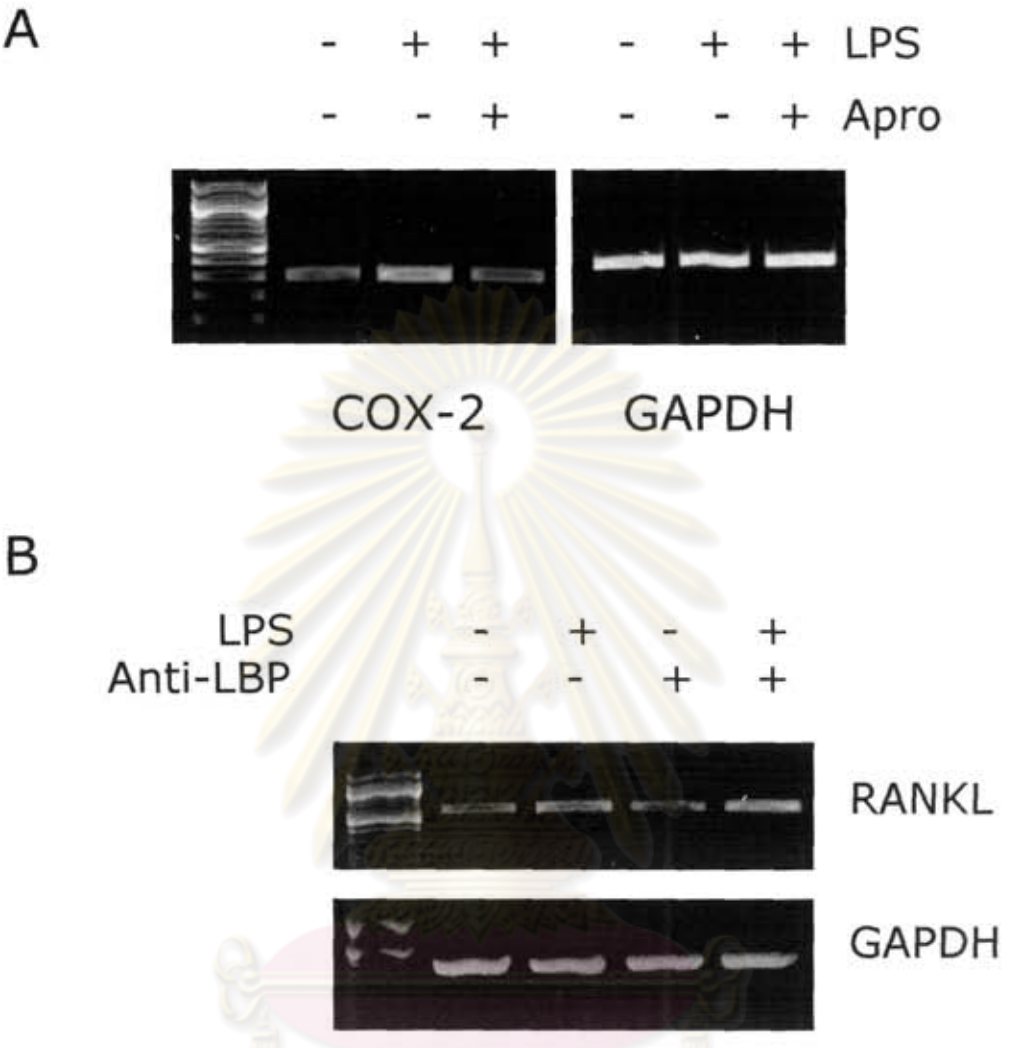

Figure 6

Figure 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).