CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The following materials were used in this study:

- Phorbol 12-myristate 13-acetate (PMA), dioctanoylphosphatidic acid (DOPA), dioctanoyl glycerol (DOG), and propanolol were purchased from Sigma-Aldrich, St. Louis, MO, USA.
- IL-1β was obtained from R&D Systems, Inc., Minneapolis, Minnesota, USA.
- The specific $cPLA_2\alpha$ inhibitor was purchased from Calbiochem, Merck Biosciences, Darmstadt, Germany.

All chemical reagents were dissolved in dimethyl sulfoxide(DMSO), and the final concentration of DMSO was less than 0.1% (vol/vol of culture medium).

- The cell wall extract of *F. nucleatum* was prepared as described previously (Krisanaprakornkit*et al*., 1998) (see detail in Appendix C).
- Ethanol, 1-butanol, and tertiary-butanol (*t*-butanol) was an analytical grade with more than 99% in purity.
- The primary antibodies for PLD1, PLD2, $cPLA_2$, MMP-2 and MMP-9 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

- The polyclonal antibody against the phosphorylated form of human cPLA$_2$ (Ser505) was obtained from Cell Signaling Technology Inc., Danvers, Massachusetts, USA.
- Nuclear and cytoplasmic extraction reagents and a protease inhibitor cocktail kit were obtained from Pierce Biotechnology, Rockford, Illinois, USA.

3.2 Cultures of human gingival epithelial cells

HGECs were isolated from gingival tissue overlying impacted third molars as described previously (Krisanaprakornkit*et al.*, 1998). Briefly, healthy gingival biopsies were obtained from the tissue overlying impacted third molar teeth of adult humans and collected in accordance with approved Human Subject policies. The tissue (the dimension was about 3 by 5 mm) was rinsed twice in HEPES-buffered saline, containing 1% penicillin, streptomycin and amphotericin B (Invitrogen™, Grand Island, New York, USA), and cut into small pieces (1 by 1 mm). The tissue explants were incubated with 0.5 mg/ml Thermolysin® (Sigma-Aldrich) in HEPES-buffered saline for 90 min at 37ºC to separate the epithelium from the underlying fibrous connective tissue.

After enzymatic separation, the epithelium was readily lifted off, and the epithelial sheets were then further incubated in 5 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA, Invitrogen™) at 37ºC for 10 min. The epithelial sheets were repeatedly pipetted to prepare a single cell suspension, and the trypsinization was stopped by addition of an equal amount of DMEM (Invitrogen™), supplemented with 10% fetal bovine serum (Invitrogen™). The cells pellets were collected and resuspended in a serum free KGM (BioWhittaker Inc., Walkersville, MD, USA),

supplemented with human recombinant epidermal growth factor, hydrocortisone, bovine insulin, bovine pituitary extract, gentamycin sulfate, amphotericin B, and low calcium concentration (0.03 mM). Resuspended epithelial cells are plated in T-25 flasks (Corning Glass Works, Corning, NY, USA), and grown in a humidified chamber at 37ºC and 5% CO_2. Culture medium was changed three times a week. After 80% confluence, HGECs were trypsinized and then passaged to new tissue culture flasks. The HGECs at passage 2-4 were used throughout this study.

3.3 Cultures of human gingival fibroblasts

HGFs were isolated from gingival tissue overlying impacted third molars as described previously (Krisanaprakornkit*et al.*, 1998). Briefly, healthy gingival explants, from which the epithelium was removed, were cut into a smaller dimension (approximately 1 by 1 mm), and placed in a 60-mm tissue culture dish (Nunc™, Roskilde, Denmark). HGFs, cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified chamber at 37ºC and 5% CO_2. Culture medium was changed twice a week. After the cells reached confluent, HGFs were trypsinized, washed twice, and then sub-cultured to new tissue culture flasks. HGFs at the fourth to eighth passage were used throughout this study.

3.4 Cell stimulation

After 80% confluence, HGECs were stimulated with 3, 10, 30 μg/ml of *F. nucleatum* cell wall extract, or 1, 10, 100 ng/ml of PMA for various times in the presence or absence of pharmacological inhibitors. In addition, HGECs were stimulated with various doses of DOPA or of DOG. Regarding HGFs, after 80% confluence, cell were stimulated with 1 ng/ml of IL-1β or 10 μg/ml of *F.*

*nucleatum*cell wall extract for indicated times, or were stimulated with various concentrations (0.01, 0.1, 1, 10 ng/ml) of IL-1β overnight.

3.5 Isolation of total RNA and RT-PCR

Total RNA was isolated with an Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instruction. Samples of total RNA were quantified by optical density reading at 260 nm. The expression of mRNAs for PLD1, PLD2, MMP-2, MMP-9, $cPLA_2$ and GAPDH was detected by means of RT-PCR. Briefly, two micrograms were used for the synthesis of cDNA by the SuperScript™ First-Strand cDNA Synthesis System (Fermentas, Hanover, MD, USA). The RT protocol was previously described (Krisanaprakornkit*et al.*, 2008), and PCR was performed in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany).

The PCR primers and their conditions were previously described for PLD1 and PLD2 (Krisanaprakornkit*et al.*, 2008), for MMP-2 and MMP-9 (Li *et al*., 2001) and for $cPLA_2$ (Dommisch*et al*., 2007). The primer sequences for MMP-2, MMP-9, PLD1, PLD2, $cPLA_2$, and GAPDH, and their predicted sizes of PCR products were summarized in Table 3.1. The conditions of PCR amplification for each pair of primers were summarized in Table 3.2. The PCR products were resolved on a 1.2% agarose gel in 1X Tris-borate EDTA (TBE) and visualized by ethidium bromide staining. Photographs of gels were taken by a charge-coupled device (CCD) camera attached to the ChemiDoc XRS (Bio-Rad Laboratories).The sizes of PCR products for the PLD1α and β splice variants, the PLD2 isoform (Diaz *et al*., 2002), and MMP-2 were as predicted, and the results from sequencing reactions at the Macrogen Inc.,

Seoul, Korea, confirmed 100% identities for MMP-9 (NM004994) and $cPLA_2$(NM024420).

Table 3.1 The summary of the sequences of primers and the amplicon sizes in base pairs (bp).

Product	Forward primer	Reverse primer	Amplicon size (bp)
MMP-2	5’-CTCAGATCCGTGGTGAGATCT-3’	5’-CTTTGGTTCTCCAGCTTCAGG-3’	496
MMP-9	5’- ATCCAGTTTGGTGTCGCGGAGC-3’	5’-GAAGGGGAAGACGCACAGCT-3’	552
$cPLA_2$	5’-CCAAGGGAAACTGAGGAAGA-3’	5’-AGGGAAACAGAGCAACGAGA-3’	223
PLD1	5’-GGGATCCGTGTGAAGCGGGTCACTTCAGGACCG-3’	5’-GGGAATTCTCTGGTTTCCCCATGCAGCTCTCCCAC-3’	446 (α), 332 (β)
PLD2	5’-GGGAATTCGACGGCGACCCCTGAGAGCCTCTTC-3’	5’-GGGAATTCACGGTATTTCTTCTTGGTTGTCCAGG-3’	329
GAPDH	5’-ACCACAGTCCATGCCATCACTGC-3’	5’-TCCACCACCCTGTTGCTGTAGC-3’	452

Table 3.2 The summary of amplification conditions for each gene.

	Denature (Temperature and Time)	Annealing (Temperature and Time)	Extension (Temperature and Time)	Number of PCR cycles
MMP-2	95 °C 40 s	60 °C 45 s	72 °C 75 s	20
MMP-9	95 °C 40 s	60 °C 45 s	72 °C 75 s	28
$cPLA_2$	94 °C 60 s	55 °C 45 s	72 °C 60 s	28
PLD1	94°C 45 s	60 °C 45 s	72 °C 45 s	30
PLD2	94°C 45 s	57 °C 45 s	72 °C 45 s	35
GAPDH	95 °C 45 s	60 °C 60 s	72 °C 85 s	20

3.6 Real-time PCR

The real-time PCR experiment was performed using 5% (vol/vol) of cDNA, prepared from 2 μg of total RNA, and the Light-Cycler-FastStart DNA Master SYBR Green I system (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (Krisanaprakornkit*et al.*, 2008). The fluorescence activity was measured at 530 nm during the extension phase. The relative induction of MMP-9 mRNA expression normalized by the level of GAPDH mRNA expression in each stimulated sample was determined by comparison with that in the control sample, which was set to 1.0. The mean relative ratio of MMP-9 to GAPDH in each sample was calculated from three separate experiments.

3.7 Western blot analyses for cytoplasmic and nuclear extracts and culture supernatants

HGECs at 80% confluence were stimulated with different doses of *F.nucleatum* cell wall extract or PMA for indicated times, and culture supernatants were collected for determination of MMP-9 secretion. Regarding HGFs, cells at 80% confluence were stimulated with 1 ng/ml of IL-1β for indicated times, and culture supernatants were collected for determination of MMP-2 secretion.

For determination of PLD1, PLD2 and $cPLA_2$, whole cell lysates were extracted in RIPA buffer (Krisanaprakornkit*et al.*, 2008), containing 50 mM HEPES (pH 7.5), 1% Nonidet P-40 (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 50 mMNaCl, 50 mMNaF, 1 mM sodium orthovanadate (Sigma-Aldrich), 1 mMnitrophenylphosphate, 10 μg/ml aprotinin (Sigma-Aldrich), 5 mMbenzamidine (Sigma-Aldrich), and 2 mM PMSF (Calbiochem). The lysates were transferred to

1.5-ml microcentrifuge tubes,vortexed vigorously, and centrifuged for 5 min at 4 °C. The protein content in the supernatant was determined by a protein assay (Bio-Rad Laboratories) with γ-globulin as a standard.

Five percent of the culture supernatant, 40 μg of total protein from whole cell lysates, or 10 μg of total protein from either the nuclear or the cytoplasmic extract were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad Laboratories) for 12 h in the cold. The membranes were blocked and incubated with primary antibody against human PLD1, PLD2, MMP-2, MMP-9, the phosphorylated form of $cPLA_2$, $cPLA_2$, or GAPDH. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (KPL, Gaithersburg, MD, USA) at 1:2000 in 10 ml of blocking buffer for 1 h at room temperature with gentle agitation, and then incubated with the LumiGLO Reserve Chemiluminescent substrate (KPL). The excess developing solution was drained off the membranes, and the signals on the membrane were captured with a cool CCD camera attached to the ChemiDoc XRS (Bio-Rad Laboratories).

Nuclear and cytoplasmic fractions were isolated with an NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instruction. Briefly, the cell supernatant was removed and the cell pellet was left as dry as possible. The ice-cold CER I was then added to the cell pellet, agitated for 15 seconds, and incubated on ice for 10 minutes. The ice-cold CER II was then added, vortexed for 5 seconds, and incubated on ice for 1 minute. The extract was centrifuged for 5 minutes, and the supernatant (cytoplasmic extract) fraction was transferred to a clean pre-chilled tube. The insoluble fraction was

resuspended with the ice-cold NER, and was alternately incubated on ice and shaken every 10 minutes, for the total of 40 minutes. Then, the supernatant (nuclear extract) fraction was centrifuged and transferred to a clean pre-chilled tube and stored at -80°C until further analyses by western blotting mentioned above.

3.8 Gelatin zymography and quantification of gelatinolytic area

The activity of gelatinase MMP-2 and MMP-9 in supernatants of HGECs and HGFs was determined by gelatin zymography as described (Steinbrenner, 2003). Zymography was performed by running aliquots of the culture supernatants under denaturing, but non-reducing conditions, in 10% SDS-PAGE, containing 0.1% gelatin. Gelatin type B (Sigma-Aldrich) was co-polymerized at a final concentration of 1 mg/ml. Thirty microliters (0.15%) of the culture supernatant from each culture well were dried and mixed with 1X sample buffer (62.5 mMTris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% (w/v) bromophenol blue) and then resolved for 200 min at 90 V along with standard molecular weight markers (Fermentas).

After electrophoresis, SDS was removed by washing with 2.5% Triton X-100 at room temperature for 15 min twice, and the gels were incubated in an activating buffer (50 mMTris-HCl, 10 mM $CaCl_2$, 50 mMNaCl, 0.05% Brij35) for 16 h at 37ºC. The gels were subsequently stained with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) for 6 h, and destained with 50% methanol and 10% acetic acid to reveal clear bands. Quantification of the gelatinolytic clear bands on the zymography was performed by densitometry using the Scion Image software for PC (Scion Corporation, Frederick, MA, USA), working in the Gel Plot 2 mode, and the image acquisition was done with a Hewlett Packard scanner.

3.9 Immunofluorescence

HGECs were grown on cover slips to 50–60% confluence. HGECs were treated with with*F. nucleatum* cell wall extract or PMA. Cells were washed three times with cold phosphate buffer saline (PBS), fixed for 15 min in 10% ice-cold paraformaldehyde in PBS. These were followed by blocking in a 3% bovine serum albumin (BSA) and PBS solution for 30 min. Thereafter, the cells were incubated at room temperature with primary antibody against phospho-$cPLA_2$ (Cell Signaling Technology Inc., MA, USA) (1:100) or $cPLA_2$ (Santa Cruz Biotechnology Inc., CA, USA) (1:100) in a PBS solution, containing 1% (w/v) of BSA for 30 min. Cells were washed five times in PBS and incubated with a conjugated secondary antibody (goat anti-rabbit or goat anti-mouse immunoglobulin G, conjugated with Fluorescein (FITC)) in a PBS solution, containing 1% (w/v) BSA for 1 h. The cover slips were mounted on glass slides with a fluorescence mounting medium. The fluorescent signals were observed under the fluorescence microscope at different magnification levels, and the fluorescence images were acquired with an attached cool CCD camera (DP71, Olympus). The fluorescence pictures were modified and combined with the software Photoshop program version 7.0.

3.10 Assays for PLD activity

After HGECs were stimulated with 10 μg/ml of *F. nucleatum* cell wall extract or 100 ng/ml of PMA for indicated times, KGM was replaced with 300 μl of ice-cold 50 mMTris-HCl, pH 8.0. HGECs were broken by three freeze and thaw cycles. Samples were collected, and 100 μl of the samples were mixed with 100 μl of the Amplex Red reaction buffer (Amplex® Red Phospholipase D assay kit, Molecular Probes, Inc.,

Eugene, OR, USA). The PLD activity was assayed in triplicate for each sample by determining the fluorescence activity after 1-h incubation at 37°C in the dark with the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VA, USA). A standard curve was performed with purified PLD from *Streptomyceschromofuscus*(Sigma-Aldrich, catalog number P8023), whose concentrations range from 0 to 500 mU/ml. The experiment was repeated three times using different cell lines derived from different donors.

3.11 Extraction and thin-layer chromatography of lipids

Total lipids from HGECs were extracted according to the method of Bligh and Dyer (1959). Phosphatidic acid was separated from other lipids by one-dimensional thin-layer chromatography (TLC) on silica gel 60 F254 aluminum-backed thin-layer plates. Dioleoyl PA from Sigma–Aldrich (catalog number P2767) was separated along with the lipid samples as a PA standard. PA was resolved from other lipids by a three-step one dimensional TLC, modified from Tou*et al*. (1999).

Briefly, the chromatogram was first developed in a solvent system consisting of ethyl acetate/acetic acid/2,2,4-trimethylpentane (9:2:5, by volume). After the chromatogram had been hood-dried for 1 h, it was cut 1 cm above the PA standard to remove the neutral lipids. It was then turned 180º and developed in chloroform/methanol/28% ammonium/water (40:20:3:1, by volume) until the solvent moved to the sample origin on the chromatogram. The chromatogram had been hood-dried for 1 h, and it was turned 180º and developed in the second solvent system. After the chromatogram had been hood-dried for 1 h, it was stained with iodine vapor for 5 min and the chromatogram pictures were scanned by a LaserJet 3020.

3.12 Statistical analysis

The differences in MMP-9 mRNA expression and secretion between the control and the stimulated samples and between the stimulated sample and the inhibitor-treated samples were expressed as mean ± standard deviation (SD) and tested by Student's *t* test at the significance level $P<0.05$ or $P < 0.01$.

3.13 Location

This study were undertaken at

1. Oral Biology Laboratory, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand.
2. Dental Research Center, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand.
3. Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
4. Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.