CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Fibroblasts were cultured from the explants obtained from the periodontal ligament (PDL) attached to non-carious, non-inflamed, freshly extracted third molars (Pattamapun et al., 2003). All fibroblasts were collected from patients who gave informed consent. The human periodontal ligament tissues were scraped out from the middle third of the root and harvested on a 35-mm culture dish (Nunc, Napervile, IL, USA). The explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Gibco BRL, Carlsbad, CA, USA) at 37ºC in a humidified atmosphere of 95% air, 5% CO_2. After reaching confluence, fibroblasts were subcultured with a ratio of 1:3. Fibroblasts from the third to the eighth passage were used. All experiments were performed in triplicate using cells prepared from three different patients.

2.2 Cultivation of bacteria

E. faecalis ATCC19433 and ATCC14506 stocks were plated on blood agar (Biomerieux, Marcy l'Etoile, France) and incubated at 37ºC. After 24 hours of incubation, a single colony was suspended in brain heart infusion broth (BHI) (Difco, Sparks, MD, USA), and incubated at 37ºC in anaerobic conditions overnight, and monitored for optical density. After the bacterial growth yield

at OD600 was 1.0, which corresponded to 10^9 colony forming units (CFU) per ml, cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4ºC. Supernatant was filtered through a 0.22 µm membrane filter. The *E. faecalis* supernatant was kept at -80°C until use.

The pellet cells were boiled for 30 minutes at 100°C. To ensure the bacteria were completely killed, the inactivated bacteria were plated on the media containing 1.5% agar and cultured overnight at 37°C. No bacterial colonies were observed. Heat-killed *E. faecalis* was kept at -80°C until use.

2.3 Cytotoxicity of *E. faecalis* supernatant and heat-killed *E. faecalis*

Cytotoxicity of *E. faecalis* supernatant and heat-killed *E. faecalis* was analyzed by MTT [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide] assay after 24 hours of treatment. Briefly, fibroblasts were seeded in 24-well plates (Nunc, USA) at a density of 50,000 cells ml^{-1} $well^{-1}$ and allowed to attach for 16 hours. The medium was replaced with serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma, St. Louis, MO, USA) for six hours. Cultured fibroblasts were treated with various concentrations of *E. faecalis* supernatant (2-40% (v/v)) and heat-killed *E. faecalis* (the ratio of the number of fibroblasts to the number of heat-killed *E. faecalis* bacteria was 1:10-1:10,000) in serum-free conditions for 24 hours. For MTT assay, the medium was replaced by fresh medium containing 250 µg/ml of MTT for the last four hours. The blue formazan product in each well was dissolved by 900 µl dimethylsulfoxide (DMSO) (Sigma, USA) and 125 µl glycine buffer and quantified by measuring its optical density at wavelength 570 nm using a DU650 spectrophotometer (Beckman, Rockville, CA, USA). The number of viable

cells was proportional to the amount of formazan production. Number of viable cells was determined by comparing the measured optical density to a standard curve generated from known cell numbers and expressed as a percentage of relative viable cell numbers comparing to control. Various concentrations of *E. faecalis* supernatant and heat-killed *E. faecalis* that did not show any toxicity were used in the next experiment.

All experiments were performed three times. Experimental values were expressed as median percentage of relative viable cell numbers. Comparisons were performed using the Kruskal-Wallis H-Test and Mann-Whitney U-test. A *p*-value less than 0.05 was considered statistical significance. All statistical analyses were performed using SPSS version 14 (SPSS Inc, Chicago, IL, USA).

2.4 Activation of cultured fibroblasts with *E. faecalis* supernatant and heat-killed *E. faecalis*

Fibroblasts were seeded in 24-well plates (Nunc, USA) at a density of 50,000 cells ml^{-1} $well^{-1}$ and allowed to attach for 16 hours. Cells were allowed six hours in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma, USA) before being treated. Non-toxic concentrations of *E. faecalis* supernatant and heat-killed *E. faecalis* were added to the culture and incubated for 48 hours. All treatments were conducted in serum-free conditions. As a negative control for supernatant stimulation, fibroblasts were cultured with 15% (v/v) of BHI. As a negative control for heat-killed stimulation, fibroblasts were cultured with medium alone. Concanavalin A (Con A) (Sigma, USA) was used as the positive control. After 48

hours, the conditioned medium was collected and kept at -20°C prior to the analysis of MMP-2 activation.

2.5 Gelatin zymography

MMP-2 activation was evaluated by gelatin zymography. Fifteen µl of the culture medium was mixed with non-reducing sample buffer and subjected to a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were gently shaken in a re-naturing buffer (0.25% Triton-X-100) at room temperature for 15 minutes, three times, to remove SDS, and then incubated in a developing buffer (0.15 M NaCl/ 10 mM $CaCl_2$/ 50mM Tris-HCl pH 7.5/ 0.1% Brij-35) at 37°C for 16-18 hours. The gels were stained with 2.5% Coomassie Brilliant Blue and de-stained to visualize the gelatinolytic protein band.

Zymographic activity was quantified by densitometric analysis, employing the Scion Image software (National Institutes of Health [NIH], Rockville, MD, USA). Intensity of band was expressed as band density (arbitrary unit).

2.6 RNA extraction

Fibroblasts were seeded in 60 mm dishes (Nunc, USA) at a density of 750,000 cells ml^{-1} $dish^{-1}$ and treated with either *E. faecalis* supernatant or heat-killed *E. faecalis*, as described above. After 48 hours, total cellular RNA was extracted with an Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. RNA samples were analyzed by DU650 spectrophotometry (Beckman, CA, USA) to determine total RNA, yield and purity (OD260:OD280 1.7–2.0). RNA samples were stored at -80°C until use.

2.7 Reverse transcription polymerase chain reaction (RT-PCR)

One ng of each RNA sample was converted to cDNA by reverse transcription using a RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). Subsequent to the reverse transcription, a polymerase chain reaction was performed.

The primers specific for MMP-2, MT1-MMP, TIMP-2 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were prepared by GENSET (Genset Biotech, Singapore). The oligonucleotide sequences of MMP-2, MT1-MMP, TIMP-2 and GAPDH primers were:

MMP-2	forward: 5′-CTCAGATCCGTGGTGAGATCT-′3,
	reverse: 5′-CTTTGGTTCTCCAGCTTCAGG-′3,
MT1-MMP	forward: 5′-CATCGC TGCCATGCAGAAGT-′3,
	reverse: 5′-GTCATCATCGGGCAGCAC-′3
TIMP-2	forward: 5′-GGAAGTGGACTCTGGAAACGACATT-′3,
	reverse: 5′-CTCGATGTC GAGAAACTCCTGCTTG-′3,
GAPDH	forward: 5′-TGAAGGTCGGAGTCAACGG AT-′3,
	reverse: 5′-TCACACC CATGACGAACATGG-′3.

The PCR was performed using 2X PCR Master Mix (Fermentas, Canada) with a PCR volume of 25 µl. The reaction mixture contained 1 µl of primers and 10 µl of RT product. The PCR working conditions were set at a denaturation for 1 minute at 94^{o}C, primer annealing for 1 minute at 60^{o}C, and chain elongation for 1.45 minutes at 72^{o}C on a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). MMP-2, MT1-MMP, TIMP-2 and GAPDH were amplified for 20, 28, 24, and 23 cycles, respectively. Products were stored at 4^{o}C until use. The amplified DNA was

then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining.

Densitometric analysis was analyzed by Scion Image software (NIH, USA). Each gene was normalized with GAPDH (internal control) and expressed as a relative density to its control.

2.8 Western blot analysis

Fibroblasts were seeded in 60 mm Petri dishes (Nunc, USA) at a density of 750,000 cells ml^{-1} $dish^{-1}$ and treated with *E. faecalis* supernatant and heat-killed *E. faecalis* at various concentrations. The conditioned medium was collected and centrifuged to remove cell debris for MMP-2 and TIMP-2 analysis. For determination of MT1-MMP, whole cell lysates were analyzed from cell extracts using lysis buffer (Krisanaprakornkit et al., 2002). The protein content in both the culture medium and in the cell lysates was determined by BCA™ protein assay (Bio-Rad, USA). MMP-2 and MT1-MMP samples, 25 µg total protein per lane, were subjected to electrophoresis under a reducing condition on 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Schleicher & Schuell, Waltham, MA, USA) for 12 hours at 4°C. TIMP-2 samples, 100 µg total protein per lane, were used for electrophoresis using a reducing condition on a 15% polyacrylamide gel. The primary antibody for MMP-2 (rabbit anti-human, the affinity-isolated antibody against the N-terminus of human MMP-2 protein) (Sigma, USA) and MT1-MMP (rabbit anti-human, the affinity-isolated antibody against the MT1-MMP hinge region) (Sigma, USA) was used at a dilution of 1:1,000 in 5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.1% (v/v) of Tween-20 (0.1% T-TBS),

activated overnight at 4^{o}C. TIMP-2 (rabbit anti-human, the affinity-isolated antibody against the C-terminal of human TIMP-2 protein) (Sigma, USA) was used at a dilution of 1:1,000 in 5% bovine serum albumin (BSA) in 0.1% T-TBS. The membranes were incubated with HRP-conjugated secondary antibody (Cell signaling Technology, Danvers, MA, USA) at a dilution of 1:2000 in 10 ml of blocking buffer for one hour at room temperature with gentle agitation, and then incubated with the LumiGLO Reserve Chemiluminescence substrate (KPL, Gaithersburg, MD, USA). The excess developing solution was drained off the membranes, and the signals were captured with a CCD camera attached to the ChemiDoc XRS (Bio-Rad, USA).

Densitometric analysis was analyzed by Scion Image software (NIH, USA). Each gene was normalized with GAPDH (internal control) and expressed as a relative density to its control