CHAPTER 1

INTRODUCTION

1.1 Principles, theory and rationale

Periodontitis is an advanced form of periodontal disease that involves destruction of alveolar bone. It is considered to be a common cause of adult tooth loss (Papapanou, 1996). One of its etiologies is specific types of Gram-negative microorganisms in dental plaque biofilm, including *Porphyromonasgingivalis* (*P. gingivalis*), *Aggregatibacteractinomycetemcomitans* (*A. actinomycetemcomitans*) and *Tannerellaforsythensis* (*T. forsythensis*) [The American Academy of Periodontology (AAP), 1996]. However, one study conducted in the Thai population has reported that the presence of only *A. actinomycetemcomitans*and *P. gingivalis*, but not *T. forsythensis*, is positively associated with chronic periodontitis (Torrungruang*et al*., 2009). To date, it has become clear that the pathogenesis of the disease is caused by the complex interactions between plaque microorganisms and host defense molecules, which are produced by both resident and infiltrating immune cells at the site of inflammation (Offenbacher*et al*., 1993; Van Dyke and Serhan, 2003). These interactions lead to the release of pro-inflammatory mediators, *e.g.*, interleukin (IL)-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-6, IL-8, matrix metalloproteinases (MMPs), and prostaglandin E_2 (PGE_2) (Kornman*et al*., 1997). High levels of these mediators have been identified in periodontitis lesions and are thought to play a pivotal role in destructive processes of periodontal tissues

(Socransky and Haffajee, 1991). However, the precise pathological mechanisms underlying tissue destruction are still not well understood.

MMPs are classified as a family of structurally related, but genetically distinct, enzymes that degrade extracellular matrix (ECM) and basement membrane (BM) components. Up until now, 25 members of the MMP family, including 22 human MMPs, have been identified and characterized (Sorsa*et al*., 2006). These proteinases are involved in a number of physiological events, such as, embryonic development, tissue remodeling, salivary gland morphogenesis, and tooth eruption. They are also involved in various pathological processes, including periodontal disease, arthritis, cancer, atherosclerosis, diabetes, pulmonary emphysema, and osteoporosis (Birkedal-Hansen *et al*., 1993). It has been reported that resident periodontal cells, including gingival fibroblasts, gingival epithelial cells, periodontal ligament fibroblasts, osteoblasts, osteoclasts, and endothelial cells, are activated in response to various pro-inflammatory stimuli to express and secrete distinct types of MMPs, which play a role in periodontal tissue destruction (Sorsa*et al*., 2004; Sorsa*et al*., 2006; Cox *et al*., 2006; Uitto*et al*., 2003; Chang *et al*., 2002). In the present study, the biology of MMP will be primarily focused on gingival fibroblasts and gingival epithelial cells due to a much larger number of these cells in periodontium in comparison to other resident cell types. Moreover, it is possible that the majority of these two resident cell types in the periodontium may influence and contribute to the increased or decreased levels of MMP enzymes, observed in a number of clinical studies.

Several previous clinical studies have shown that the expression levels of MMP-8, MMP-9, and MMP-13 are raised in accordance with increased severity and activity of

periodontal disease, whereas these levels decline after periodontal treatment (Birkedal-Hansen, 1993; Golub*et al*., 1994; Uitto*et al*., 2003; Sorsa*et al*., 2004; Kivelä-Rajamäki*et al*., 2003), suggesting the association between expression of some members of the MMP family and the degrees of alveolar bone loss in periodontal disease. Furthermore, some studies showed that MMP-2 and MMP-9 are involved in the gingival extracellular matrix degradation during the progression of periodontitis (Dong *et al*., 2009; Ejeil*et al*., 2003; respectively). Interestingly, pro-MMP-2 is expressed in clinically normal and diseased tissues, whereas only the active form of MMP-2 has been detected in biopsies obtained from patients with periodontal disease (Korostoff*et al*., 2000). Collectively, all of these studies emphasize the significance of MMPs, especially MMP-2 and MMP-9, in the pathogenesis of periodontal disease.

Consequently, it is essential to study the biology, particularly in the aspects of expression and regulation, of MMP-2 and MMP-9 enzymes in order to better understand their roles in the pathogenesis of periodontal disease. In this study, a group of gelatinase enzymes that include MMP-2 and MMP-9, previously called gelatinase A and gelatinase B, respectively, is selected for a thorough investigation into their expression and regulation in primary gingival epithelial cells and in primary gingival fibroblasts due to their crucial role in the pathogenesis of periodontal disease, as mentioned above.

It is well known that MMP-9 is primarily synthesized and secreted from resident gingival epithelial cells and skin keratinocytes (Mäkelä*et al*., 1994 and Sawicki*et al*., 2005, respectively) in addition to infiltrating immune cells, like neutrophils and T-lymphocytes, whereas resident gingival fibroblasts have the ability to synthesize and secrete MMP-2 in pro-form (pro-enzyme). The pro-enzyme of MMP-2 requires further

activation for its catalytic activity by the formation of a trimolecular complex, consisting of MT1-MMP, TIMP-2, and MMP-2 on the cell surface (Stronging*et al*., 1995). Moreover, there have been a number of studies that show the up-regulation of MMP-9 expression by various pro-inflammatory stimuli in different types of primary human cells and tumor cell lines, yet there have been much fewer studies regarding the detailed molecular mechanisms of MMP-9 up-regulation, especially some important signal transducers in controlling the expression, secretion, and activity of MMP-9 in primary human gingival epithelial cells (HGECs). For example, some previous studies have shown a critical role of phospholipase D (PLD) and phospholipase A_2 (PLA_2) as signaling molecules to regulate MMP-9 expression in tumor cell lines (Willige*et al*., 1999; Ho *et al*., 2003; Gorovetz*et al*., 2006; 2008).

Furthermore, some resent studies in tumor cell lines have demonstrated that the increased expression of PLD enhances MMP-2 expression (Reich *et al*., 1995; Park *et al*., 2009) and that the secretory phospholipase A_2 induces pro-MMP-2 activation (Lee *et al*., 2006). However, to the best of my knowledge, there has never been any study to show the regulation of MMP-2 by PLD and secretory PLA_2 in human gingival fibroblasts (HGFs).

Therefore, it is interesting to further investigate the role of some previously-reported molecules that are involved in MMP-2 and MMP-9 regulation in both HGFs and HGECs. Particularly, it is interesting to determine the role of two enzymes, including PLD and PLA_2, which specifically cleave some phospholipid molecules that are constituents of eukaryotic cell membrane, in MMP-2 and MMP-9 regulation in both HGFs and HGECs. It is believed that deep insight into the molecular biology of MMP-2 and MMP-9 with an

emphasis on the regulation of their synthesis, secretion, and activity, will be beneficial for our understandings in the pathogenesis of periodontal disease. Furthermore, we wish that the knowledge gained from this study will be useful for designing novel therapeutic modalities that aim to antagonize some specific signaling molecules that control MMP-2 and MMP-9 expression and activity.

1.2 Objectives

In this study, an *in vitro* model of HGFs and HGECs will be used

1. To determine the expression and activity of MMP-2 in response to treatment with IL-1β, a pro-inflammatory cytokine, or with the cell wall extract of *Fusobacteriumnucleatum* (*F. nucleatum*), one of the periodontopathogenic bacteria, in HGFs.

2. To examine the expression and activity of MMP-9 upon treatment with the cell wall extract of *F. nucleatum*, or with phorbol 12-myristate 13-acetate (PMA), a potent epithelial activator, in HGECs.

3. To characterize the role of some signaling proteins, especially PLD and PLA_2, in the regulation of gelatinase activity in both cell types.

1.3 Hypotheses

1. MMP-2 is expressed in primary HGFs at both messenger ribonucleic acid (mRNA) and protein levels, and the secretion of MMP-2 can be detectable in cell-free culture supernatant.

2. MMP-2 expression and activity are up-regulated by IL-1β or *F. nucleatum* cell wall extract in HGFs in a dose-dependent manner.

3. MP-9 is expressed in primary HGECs at both mRNA and protein levels, and the secretion of MMP-9 can be detectable in cell-free culture supernatant.

4. MMP-9 expression and activity are up-regulated by *F. nucleatum*, a Gram-negative periodontal bacterium, as well as PMA, a well-known epithelial activator, in HGECs in a dose-dependent manner.

5. PLD and PLA_2 play a critical role in regulating MMP-2 and MMP-9 expression in primary HGFs and HGECs, respectively, similar to their role in regulating MMP-2 and MMP-9 expression as previously reported in some tumor cell lines.

1.4 Education/application advantage

To address the hypotheses in this proposal, several advantages from expertise in the biology of HGFs and HGECs and thorough knowledge in innate immune response, particularly the effector molecules in innate immunity, like cytokines, chemokines, and antimicrobial peptides, have been taken. In addition, several molecular biology techniques that are necessary for most of the experiments proposed in this proposal are routinely conducted at the Oral Biology Laboratory, Faculty of Dentistry, ChiangMai University. Moreover, the use of cell wall extract of *F. nucleatum*, one of the natural stimulants used in this study, provides more consistent results between experiments in that the use of live or whole periodontal bacterial cells as natural stimulants is too difficult to manage and can possibly create a lot of variations in result interpretation.

We wish that the knowledge gained from this study will be beneficial for better understanding the regulation of several other effector molecules in innate immunity, in addition to MMP-2 and MMP-9, such as cyclooxygenase (COX)-2, human β-defensins, etc., and can thus be used as an *in vitro* model to study the regulation and signal transduction pathway(s) of these molecules.

1.5 Research designs, scope and methods

In this study, an *in vitro* culture model of HGECs and HGFs will be used to test the hypotheses as follows:

1. MMP-2 is expressed in primary HGFs at both mRNA and protein levels, and the secretion of MMP-2 can be detectable in cell-free culture supernatant.

To study MMP-2 expression and up-regulation in HGFs, we will first investigate MMP-2 expression at the mRNA level by reverse transcriptase-polymerase chain reaction (RT-PCR) (see Materials and Methods for details). Moreover, the MMP-2 activities will be investigated in cell-free culture supernatant by gelatin zymography (see Materials and Methods for details). Cultured HGFs are grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Once the cells are 80% confluent, HGFs are starved from fetal bovine serum for 6 hours prior to stimulation with IL-1β in dose- and time-dependent fashions. Total RNA and culture medium will be collected for further analyses as mentioned above.

2. MMP-9 is expressed in HGECs at both mRNA and protein levels, and the secretion of MMP-9 can be detected in cell-free culture supernatant. MMP-9 expression and activity are up-regulated by the cell wall extract of *F. nucleatum*, a Gram-negative periodontal bacterium, as well as PMA, a well-known epithelial activator, in dose- and time-dependent manners.

To study MMP-9 expression and up-regulation in HGECs, we will first investigate MMP-9 expression at mRNA level by RT-PCR (see Materials and Methods for details). Moreover, we investigated the expression of MMP-9 protein and its activity in cell-free culture supernatant by western blot hybridization and gelatin zymography, respectively (see Materials and Methods for details). Cultured HGECs are grown in serum-free keratinocyte growth medium (KGM). Once the cells are confluent, they are stimulated with either the cell wall extract of *F. nucleatum* or PMA in dose- and time-dependent fashions. Total RNA, cell lysates, and culture medium will be collected for further analyses as mentioned above.

3. Both PLD and cytosolic PLA_2 ($cPLA_2$) play a critical role in regulating MMP-2 and MMP-9 expression and secretion in HGFs and HGECs, as previously reported in some tumor cell lines and human monocytes.

To determine the involvement of $cPLA_2$ and PLD in MMP-2 and MMP-9 expression, we will first examine the expression for PLD, both PLD1 and PLD2, and $cPLA_2$, at both mRNA and protein levels by RT-PCR and western blot hybridization,

respectively. The enzymatic activity of PLD is analyzed by a fluorogenic substrate in the Amplex® Red Phospholipase D assay kit (see Materials and Methods for details). In addition, a thin-layer chromatography (TLC) will be conducted to determine the lipid products, derived from the PLD enzymatic activity. Moreover, the expression and localization of a phosphorylated (or an active) form of $cPLA_2$ will be investigated by western blot hybridization and immunofluorescence, respectively (see Materials and Methods for details). Subsequently, the studies using pharmacological inhibitors, specifically blocking PLD or $cPLA_2$, are conducted. Cells are cultured as described above. Confluent cells are stimulated with stimulants as mentioned above in the presence or absence of the specific inhibitors against PLD or $cPLA_2\alpha$. All of pharmacological inhibitors are purchased from either Sigma-Aldrich or Calbiochem, USA. The mRNA and protein expression as well as the activity of MMP-2 and MMP-9 will be analyzed by RT-PCR, western blot hybridization, and gelatin zymography.