CHAPTER 5

DISCUSSION

MMP-9 expression and then secretion is induced by many bacteria in periodontal lesions and the up-regulation of MMP-9 is involved in the pathogenesis of periodontal disease, which leads to the destruction of periodontium. This study has shown that the cell wall extract of *F. nucleatum* induces *de novo* expressionofMMP-9 in HGECs, whereas it has been previously shown that whole cells of*F. nucleatum* induce secretion and activation of MMP-9 fromneutrophil granules without affecting its *de novo* expression(Ding *et al*., 1997). This is consistent with the results from an earlier study that show the process of PMN degranulation, in which the secreted phospholipases upon stimulation with a purified bacterial pathogen efficiently induce PMN degranulation, whereas the secreted phospholipases instead induce MMP-9 expression in oral epithelial cells (Ding *et al*., 1995). The rapid response by PMN degranulation and the amounts of enzymes released strongly indicate that a direct interaction between microbial whole cells and PMNs may be one of the major pathological mechanisms in periodontitis. Furthermore, the different responses between PMNs and oral epithelial cells to bacteria in terms of MMP-9 production and secretion may suggest the distinct signaling pathways in regulating induction and/or activation of MMP-9 between these two cell types.

In contrast to MMP-9 up-regulation, MMP-2 expression in HGECs is not altered upon stimulation with the cell wall extract of *F. nucleatum* or PMA, a potent

epithelial activator. This is consistent with a previous result in normal human epidermal keratinocytes (NHEK), which shows the increased MMP-9 activity in TPA-stimulated NHEK compared with untreated control cells, whereas MMP-2 expression and activity are not affected (Steinbrenner *et al*., 2005).

With respect to expression of MMP-2 and MMP-9 in HGFs, it was demonstrated in this study that HGFs express low levels of MMP-9, while they constitutively express MMP-2 mRNA. This finding is comparable to the constitutive expression of MMP-2 in endothelial cells (Nguyen *et al*., 2001). Interestingly, the protein expression and the activity of both latent and active forms of MMP-2 are accumulated and induced even in the absence of stimulation, suggesting the stability of MMP-2 protein and an autocatalytic process of MMP-2 enzyme. The autocatalytic processing of MMP-2 may possibly be achieved by a proposed self-activation of pro-MMP-2 that takes place on the cell surface by a trimolecular complex, consisting of MT1-MMP, MMP-2, and TIMP-2, and this complex has previously been detected on the surface of human gingival fibroblasts (Bolcato-Bellemin *et al*., 2000; Oyarzún *et al*., 2010).

Taken together, all of the data obtained from the studies of MMP-2 and MMP-9 expression and activities in HGFs and in HGECs reveal that both *F. nucleatum* cell wall extract and PMA up-regulate MMP-9 expression (at both mRNA and protein levels) and its gelatinolytic activity in HGECs as well. Furthermore, MMP-2 expression in HGECs remains low and unchanged upon stimulation with both stimulants mentioned above. In addition, expression of MMP-2 mRNA is constitutive in HGFs, whereas the released MMP-2 protein is accumulated with a suggestive auto-activation of pro-MMP-2 to the active form of MMP-2 even in the absence of stimulant.

It is worth noting for the kinetic study that HGECs were stimulated by both stimulants for indicated times, and the total RNA or total protein and cell-free culture supernatants of control HGECs were collected at 24 hours, i.e., at the same time point as all stimulated samples. Therefore, the cell number of HGECs should be similar between control and stimulated samples during the period of harvesting total RNA and protein. Furthermore, in order to normalize the gelatinase activity in cell-free culture supernatants, an equal volume (5% vol/vol) of cell-free culture supernatants from control and stimulated HGECs was used for either western blot analysis or gelatin zymography. This means that the total protein content within the culture supernatant is equivalent among different samples. In contrast, in HGFs, cells were serum starved for 6 hours before being stimulated by both stimulants at the same time. Subsequently, the total RNA or total protein and cell-free culture supernatant were collected at different time points. Therefore, it is likely that in HGFs, the number of HGFs should be different between different samples, and the accumulation of MMP-2 activity seen in Fig.4.11 result from the increased number of HGFs due to cellular proliferation during the prolonged period of incubation rather than the accumulation of stable MMP-2 protein. Consequently, this issue is needed to be further addressed.

The use of cell wall extract of *F. nucleatum* strain ATCC 25586 as a cell stimulant in this study is logical, since it provides more consistent results between experiments. Nevertheless, this cell wall extract is a crude extract from one reference strain of periodontal bacterium, in which it can contain a number of microbial membrane components, such as, lipopolysaccharide, peptidoglycan, etc., which can have a potential to stimulate both HGECs and HGFs. Therefore, it remains to be further elucidated which components of this microbial cell membrane are active in the

future. However, if the clinical strains of *F. nucleatum* were used to stimulate either HGFs or HGECs, some differences in terms of the induction of gelatinase activity would be seen among different bacterial subspecies isolated from clinical settings, which may possess varying degrees of virulence factors that will ultimately and inevitably affect the results of this study. Moreover, it would be very difficult to obtain the reproducibility in terms of the results between different batches of bacterial isolates.However, the doses of PMA used in this study were previously used and had no toxicity to human keratinocytes (Companjen *et al*., 2000; Zhang *et al*., 2000).

With regard to the above conclusions relating to the induction of MMP-9 expression and activity in HGECs, it is, therefore, interesting to additionally investigate the underlying mechanisms of MMP-9 mRNA and protein induction in response to the periodontal bacterium, *F. nucleatum*, as well as PMA in HGECs. I first began to study the involvement of PLD in MMP-9 up-regulation, since a few earlier studies have suggested a critical role of PLD as a signaling molecule in regulating MMP-9 expression in some tumor cell lines (Williger *et al*., 1999; Ho *et al*., 2003). Thus, PLD expression and its activity in HGECs were first examined in this study.

Two related polyphosphoinositol-activated PLD isoforms have been cloned and known as PLD1 (120 kDa) (Hammond *et al*., 1995) and PLD2 (106 kDa) (Colley *et al*., 1997), and the PLD1 consists of two splice variants, PLD1α and PLD1β (Hammond *et al*., 1997). To the best of my knowledge, there has never been any prior studies, showing mRNA and protein expression for PLD1 and PLD2 in primary gingival epithelial cells. In this study, the constitutive mRNA expression for PLD1α and β splice variants and the inducible mRNA expression for PLD2 isoform by PMA

in HGECs are demonstrated. Moreover, it is found that the PLD1β splice variant is preferentially expressed in HGECs, consistent with its sole expression in the human keratinocyte cell line HaCaT (Müller-Wieprecht *et al*., 1998). However, expression of the PLD1α splice variant is seen only in HGECs, reflecting a difference between primary cells and an immortalized cell line.

The presence of PLD1 fragments at lower molecular weights than expected suggests post-translational modification of the PLD1 protein by proteolytic cleavage. The full length form of PLD1 at 120 kDa as well as its possible fragment at 80 kDa are detected in the HGEC whole cell lysates, and this lower band may be comparable with the presence of the lower bands below 120 kDa detected in the HaCaT lysates (Müller-Wieprecht *et al*., 1998).

Furthermore, the PLD product, PA, is found in HGECs stimulated with *F. nucleatum* cell wall extract from 3 to 24 hours. Whereas PA mass is not found in control HGECs detected by thin layer chromatography. Interestingly, PA mass is still detected after stimulation for 24 hours (Figure 4.15), although the PLD activity is significantly reduced by that period of time (Figure 4.14). The possible explanation for this is because DAG kinases can generate PA from DAG (Nanjundan and Possmayer, 2003) in addition to PLD enzymes that generate PA from phosphatidylcholine.

Since ethanol and 1-butanol are preferentially used over water as the nucleophile by 1000-fold or more (Yang *et al*., 1967; Frohman *et al*., 1999), they were then used as PLD inhibitors in this study. By contrast, the tertiary alcohol, which is not an acceptor of the phosphatidyl-group, was thus used as a negative inhibitor. The results from this study show that both ethanol and 1-butanol inhibit MMP-9 mRNA

expression and activity. Notably, 1% (vol/vol of culture medium) of 1-butanol almost completely abolishes MMP-9 mRNA and activity, induced by *F. nucleatum* and PMA.

In addition, pretreatment with propranolol, the PAP inhibitor, inhibits MMP-9 mRNA and activity in HGECs, which indicates the importance of both PA and DAG, two products that are generated by PLD and PAP enzymes, respectively, in MMP-9 up-regulation. This also suggests the involvement of PLD in MMP-9 up-regulation in HGECs, similar to its involvement in MMP-9 up-regulation in human neutrophils and some cell lines (Tou and Gill, 2005; Hu and Exton, 2005).

Moreover, a product that is derived from the PLD catalytic reaction, *i.e.*, DOPA, can induce MMP-9 expression and activity by itself regardless of the stimulation with *F. nucleatum* cell wall extract or PMA. Furthermore, DOG, commonly used for DAG, can induce MMP-9 mRNA expression and activity even in the absence of stimulants. Taken together, all of these results confirm the importance of PLD pathway in MMP-9 regulation. Thus, the data obtained from this study provide compelling evidence that PLD, especially PLD1, is involved in up-regulation of MMP-9 expression and activity by *F. nucleatum* cell wall extract and PMA in HGECs.

In addition to the critical role of PLD in MMP-9 up-regulation, I further studied the involvement of $cPLA_2$ in MMP-9 up-regulation, since it has been previously shown that $cPLA_2$ is essential for regulating MMP-9 expression and activity in several cell types (Shankavaram et al., 1998; Ii et al., 2008). It is demonstrated in this study that the $cPLA_2\alpha$ mRNA expression is constitutive in HGECs, but the $cPLA_2\alpha$ protein can be activated by phosphorylation upon stimulation with *F. nucleatum* cell wall

extract or PMA. The different profiles of phosphorylation of $cPLA_2$ by these two stimulants suggest possible distinct signaling pathways used to mediate MMP-9 up-regulation between these two stimulants. To my knowledge, my study is the first to show the expression and activation of $cPLA_2$ in human gingival epithelial cells.

Furthermore, in this study, $cPLA_2\alpha$ is proven to be critical for up-regulatingMMP-9 expression and activity. This is demonstrated by the finding that pretreatment with 1 μM of the specific $cPLA_2\alpha$ inhibitor almost completely abrogates induction of MMP-9 expression and activity by *F. nucleatum* cell wall extract or by PMA. Correspondingly, 1 μM of the same specific $cPLA_2\alpha$ inhibitor can also inhibit arachidonic acid release (Ghomashchi *et al*., 2001). The results in non-immune cells obtained from this study are consistent with the findings from previous studies in immune cells, including human monocytes (Shankavaram *et al*., 1998) and mouse macrophages (Ii *et al*., 2008). Although the effective inhibitory concentrations of the specific $cPLA_2\alpha$ inhibitor for MMP-9 induction in this study are slightly higher than the sub-micromolar concentrations used in some other *in vitro* studies (Ghomashchi *et al*., 2001; Flamand *et al*., 2006), there are no significant differences between control and inhibitor-treated cells, in the aspects of cellular morphology and total RNA yield. Furthermore, GAPDH, a housekeeping gene, is equally expressed in all samples.

PMA substantially up-regulates MMP-9 expression, and its remarkable gelatinolytic activity is observed in cell-free supernatants, whereas *F. nucleatum* moderately induces MMP-9 expression and activity. The difference in terms of the levels of induction between PMA and *F. nucleatum* may be partly explained by prolonged activation of $cPLA_2$*via* phosphorylation by PMA and/or different kinetic profiles of $cPLA_2$ activation between two distinct stimulants.

The localization of the phosphorylated form of $cPLA_2$ in the nucleus suggests its role as a transcription factor, which was similarly observed in a previous study (Parhamifar *et al*., 2005). Consequently, it is possible that $cPLA_2$, particularly $cPLA_2\alpha$, may control MMP-9 transcription in conjunction with AP-1, which was previously reported to be critical for MMP-9 up-regulation (Cho *et al*., 2007) via activation of p38 MAP kinase (Simon *et al*., 2001) in *F. nucleatum*-treated gingival epithelial cells. This possibility needs to be further verified.

MMPs are generally not stored in most cell types, except neutrophils and macrophages. They are synthesized and immediately released into the extracellular matrix (Woessner, 1991). In human gingival epithelial cells, MMP-9 protein is found to be rapidly released into culture medium after being synthesized, since the levels of MMP-9 protein expression in whole cell lysates remain constant upon stimulation (data not shown). This is in agreement with the findings of a previous study (Ruhul Amin *et al*., 2003), which demonstrated that intracellular levels of MMP-9 protein remained unchanged after IL-1β stimulation, due to a well-coordinated process between MMP-9 production and secretion.

Collectively, the results from these signaling studies demonstrate two intracellular signal transduction pathways, leading to the enhanced production of MMP-9 in HGECs, induced by *F. nucleatum* cell wall extract or PMA. These two pathways include a cascade of events initiated by PLD and $cPLA_2\alpha$. Firstly, stimulation by *F. nucleatum* cell wall extract or PMA can enhance the activity of PLD enzymes, leading to the increased production of PA (Figure 5.1). PA can be further catalyzed to DAG by PAP enzymes or probably reversed back to PA by DAG kinases, causing an elevation of MMP-9 production in HGECs (Figure 5.1).

Secondly, *F. nucleatum* cell wall extract or PMA also stimulate HGECs through another signaling pathway, i.e., the cPLA$_2\alpha$ pathway, by phosphorylation of cPLA$_2\alpha$. The activation of cPLA$_2\alpha$ may lead to the increased production of AA from PC that then possibly results in an elevation of PGE_2 levels via activated COX-2 (Figure 5.1). It is likely that the secreted PGE_2 can bind to its cognate receptor, *i.e.*, EP4, resulting in an increase in the intracellular cAMP levels and MMP-9 up-regulation (Figure 5.1). However, further studies are needed to elucidate the involvement of several downstream molecules, including AA, COX-2, PGE_2, EP4, and cAMP in up-regulation of MMP-9 expression and activity in HGECs induced by *F. nucleatum* cell wall extract or PMA.

In summary, the present study demonstrates that, in HGECs, activation of PLD as well as cPLA$_2\alpha$ is critical for MMP-9 mRNA induction in response to the periodontal bacterium, *F. nucleatum*, as well as PMA, and that PLD and cPLA$_2\alpha$ act as the important signaling molecules for MMP-9 up-regulation. In contrast, MMP-2 expression is constitutive in both primary gingival fibroblasts and primary gingival epithelial cells, although a process of auto-activation of MMP-2 in gingival fibroblasts is proposed from the findings of this study. Based on all of these findings, targeted inhibition of either PLD, cPLA$_2\alpha$, or both may be therapeutically beneficial for the management of oral mucosal inflammatory disease, whose pathogenesis is involved in increased MMP-9 production, such as, periodontal disease, oral lichen planus, etc.

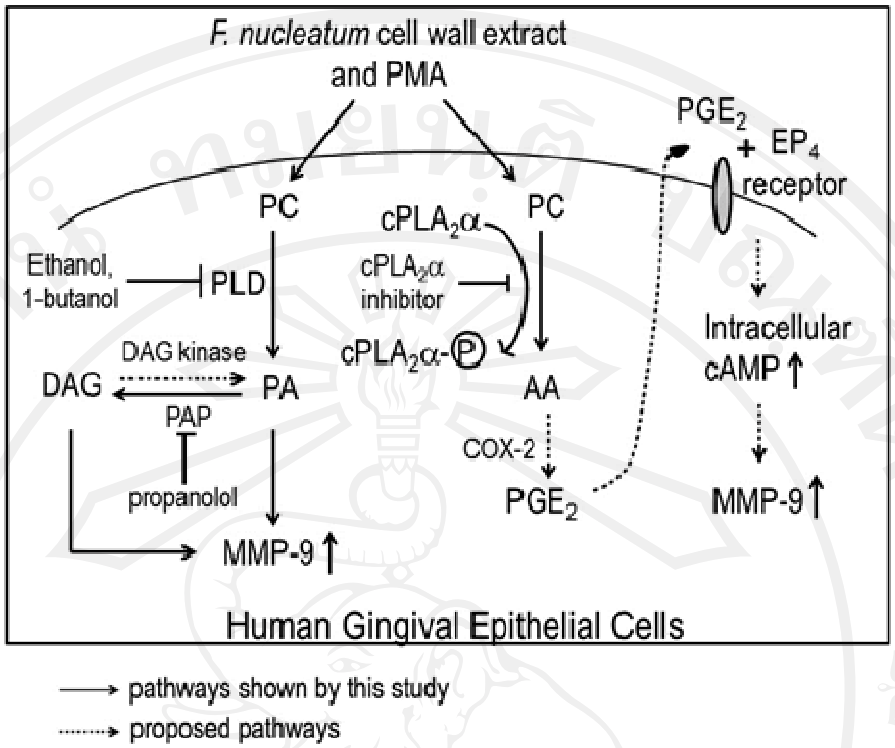

Figure 5.1 A schematic diagram shows the importance of PLD and cPLA$_2\alpha$ in MMP-9 up-regulation in HGECs. *F. nucleatum* cell wall extract or PMA can stimulate HGECs by hydrolysis of PC through PLD and cPLA$_2\alpha$. Firstly, PLD hydrolyses PC to yield PA, which induces MMP-9 up-regulation. Moreover, PA can possibly be generated from DAG by DAG kinases, resulting in an accumulation of PA in the cells that leads to prolonged induction of MMP-9. Secondly, the activated cPLA$_2\alpha$ enzyme by phosphorylation can generate AA from PC, leading to an elevation of PGE_2 via COX-2. The resulting released PGE_2 may bind to its cognate receptor, EP4, resulting in MMP-9 up-regulation via elevated levels of cAMP.