# CHAPTER 2

# LITERATURE REVIEW

Thisreview is dividedinto seven parts as follows:

2.1 Immunopathogenesis of periodontal disease

2.2 Human gingival epithelial cells (HGECs)

2.3 Human gingival fibroblasts (HGFs)

2.4 Matrix metalloproteinases (MMPs)

2.5 *Fusobacteriumnucleatum* (*F. nucleatum*)

2.6 Phospholipase D (PLD)

2.7 Phospholipase $A_2$($PLA_2$)

## 2.1 Immunopathogenesis of periodontal disease

The periodontium, a tooth supporting structure, consists of gingiva, cementum, periodontal ligament, and alveolar bone. Periodontal disease is a chronic microbial infection, and the dental plaque biofilm has been well recognized as one of its etiologic agents. The disease initiation and progression result from host responses to specific periodontal microorganisms in plaque biofilm. In healthy periodontal tissue, a low quantity of Gram-positive aerobes and facultative anaerobes, such as *Streptococcus* and *Actinomyces* species, is found in the supragingival plaque (Moore and Moore, 1994). More accumulation of plaque biofilm leads to gingival inflammation (or gingivitis) with an increase in inflammatory cell infiltration.

T lymphocytes are the dominant cell type in gingivitis lesions, reflecting the chronic nature of inflammation. In contrast, in a more advanced form of periodontal disease, *i.e.*, periodontitis, numerous T and B lymphocyte infiltrates are observed together with high levels of pro-inflammatory mediators, such as, IL-1β, TNF-α, $PGE_2$, IFN-γ, in gingival tissues and gingival crevicular fluid (Page *et al*., 1997). B lymphocytes and plasma cells are the dominant cell types in the periodontitis lesions, indicating a shift from the cell-mediated immune response, characterized by the predominant infiltration of T-lymphocytes in gingivitis lesions, to the humoral-mediated immune response of periodontitis that is mainly involved with antibody production as a result of the predominant infiltration of B-lymphocytes and plasma cells in periodontitis lesions.

Moreover, there is a shift of microbial compositions in the plaque biofilm from Gram-positive aerobes and facultative anaerobes in gingivitis to Gram-negative anaerobes in periodontitis (Seymour, 1993). The differences in microbial compositions and quantities between healthy/gingivitis and periodontitis, therefore, influence the local host immune responses. Key periodontal pathogens, such as, *P.gingivalis*, *A.actinomycetemcomitans*and *T.forsythensis* that are frequently found in deep periodontal pockets, are well recognized for their virulence factors as main etiologic agents in human periodontitis [The American Academy of Periodontology (AAP), 1996]. The binding of these pathogenic microorganisms or bacterially-derived toxins to their specific receptors on host cells leads to cell activation and release of pro-inflammatory mediators that contribute to the local inflammatory responses and periodontal tissue destruction.

2.2 Human gingival epithelial cells (HGECs)

Epithelial cells, or keratinocytes, are the major cellular component of the epithelial layer of skin and oral mucosa, and act as sensors during microbial infections (Kagnoff and Eckmann, 1997), generating and transmitting signals between microorganisms and the adjacent underlying immune cells of periodontium. Human attached gingival epithelium, a barrier facing microbial challenges, is a densecornified epithelium that has a protective surface, while the junctional region of the gingival epithelium are non-differentiated, stratified squamous epithelium with a very high rate of cell turnover. There is no keratinizing epithelial cell layer at the free surface of thejunctional epithelium that can function as a physical barrier (Nanci and Bosshardt, 2006; Bosshardt and Lang, 2005).

Among their functions, various types of epitheliacan provide a first line of defense between a microorganism and environment. Disruption of this barrier leads to microbial invasion and subsequent inflammation. In response to certain microorganisms, epithelial cells express several pro-inflammatory cytokines and chemokines, *e.g.*, IL-8, a potent chemoattractant and activator of neutrophils, monocytes, and T lymphocytes (Baggiolini*et al*., 1989; 1994). These cytokines and chemokines initiatetransendothelial migration of recruited neutrophils and monocytes/macrophages to induce inflammatory responses at the local site (Uitto*et al*., 2003; Sorsa*et al*., 2004).This suggests an additional role in the innate host defense of gingival epithelial cells (Svanborg*et al*., 1999). Thus, the interaction between epithelial cells and microorganisms is the most important step at the initial stage of inflammation (Darveau*et al*., 1998; Huang *et al*., 1998).

The next stage of *circulusvitiosus* of periodontal inflammation follows; resident periodontal cells, includingperiodontal ligament fibroblasts, gingival fibroblasts, gingival epithelial cells/gingival keratinocytes, osteoblasts, osteoclasts, and endothelialcells, are activated in response to various pro-inflammatorystimuli to express and secrete pro-inflammatorycytokines, cysteine proteinases, and matrix metalloproteinases(MMPs) (Sorsa*et al*., 1995; Ding *et al*., 1996;1997; Chang *et al*., 2002; Uitto*et al*., 2003;Sorsa*et al*., 2004; Sorsa*et al*., 2006;Cox *et al*., 2006).

2.3 Human gingival fibroblasts (HGFs)

Fibroblasts are the dominant resident cells, which inhabit the periodontal tissues. As such, they are crucial for maintaining the connective tissues, which support and anchor the tooth. In the past, they had been considered to function as the simple supporting framework for other cell types. At present, it is firmly established that fibroblasts perform several functions beyond matrix deposition. They have been found to be a dynamic cell type involved in many local tissue functions and in host defense (Phipps *et al*., 1997). They secrete collagenase and are active in matrix degradation (Trabandt*et al*., 1995). By joining with other cells in the periodontal tissue, which expressed many pro-inflammatory molecules, gingival fibroblasts can function as accessory cells in promoting inflammation and the immune response.

Gingival fibroblasts also function as a regulator of the cytokine network in periodontal tissues because they produce several kinds of cytokines when challenged by inflammatory cytokines, such as, IL-1, or bacterial components (Takada *et al*., 1991). These cells express a variety of membrane and intracellular receptors, making the cells sensitive to regulation by many physiological and pathological, paracrine and

endocrine signaling molecules (Müssig*et al*., 2005). The cells also exhibit several heterodimeric cell membrane proteins, called 'integrins', which serve as binding sites for certain extracellular matrix proteins. Most interestingly, binding to integrins results in intracellular signaling, a mechanism by which fibroblasts become sensitive to the surrounding matrix and environment (Green and Yamada, 2007; Legate *et al*., 2009). The binding to the surrounding matrix and environment can then stimulate gingival fibroblasts to secrete various soluble mediators of inflammation, including $PGE_2$, IL-1, IL-6, IL-8, and MMPs. These fibroblast-derived mediators are thought to play an important role in the inflammatory response in local periodontal lesions.

IL-1 is mainly a pro-inflammatory cytokine that stimulates the expression of many genes associated with inflammation and autoimmune disease, including those encoding cyclooxygenases, type 2 phospholipase A and inducible nitric oxide synthase (Dinarello, 2002). In the early 1980s, IL-1 was cloned and two related, but distinct, proteins – IL-1α and IL-1β – were found (Dinarello, 1994). IL-1α and IL-1β are both synthesized as 31 kDa precursors. However, large differences in post-translational processing exist because pro-IL-1α is biologically active and is only cleaved to a small extent and mainly remains intracellularly in its proform. Pro-IL-1β needs to be proteolytically cleaved and activated intracellularlly by an enzyme originally called IL-1β-converting enzyme, which is now known as caspase-1. Cleaved IL-1β is then released from the cells and can bind to IL-1 receptors (Liu*et al*., 2010).

In periodontal inflammation, IL-1β is mainly expressed by macrophages and dendritic cells, but gingival fibroblasts, periodontal ligament cells and osteoblasts can

secrete IL-1β, as well as IL-1α and IL-1 receptor antagonist, and these molecules can be found in gingival crevicular fluid (GCF) (Holmlund*et al*., 2004). The involvement of IL-1β in periodontal tissue breakdown is also indicated by observations that a functional single nucleotide polymorphism in the IL-1β gene has been associated with periodontitis. However, not all studies are conclusive in this respect, and in many studies the results are based upon small sample sizes (Huynh-Ba *et al*., 2007).

Periodontal disease is initiated by bacterial plaque and characterized by inflammation that leads to periodontal attachment loss and bone destruction. Now, it is recognized that during active periodontitis, degradation of gingival tissue (mainly collagen) is due in part to MMPs expressed *in situ* by inflammatory cells (monocytes, macrophages, lymphocytes, polymorphonuclear cells) and resident cells (fibroblasts, epithelial cells, and endothelial cells) (Reynold*et al*., 1994). A number of studies have reported the presence of cytokines produced and released from resident and inflammatory cells, particularly IL-1β. This cytokine amplifies the inflammatory process by stimulating MMP production from resident gingival fibroblasts (Meikle*et al*., 1989; Richards and Rutherford, 1990; Havemose-Poulsen and Holmstrup, 1997).

In addition, IL-1β has been shown to stimulate plasminogen activator in gingival fibroblasts (Mochan*et al*., 1986), resulting in the generation of plasmin, a well-known activator of several MMPs, particularly collagenases (Werb*et al*., 1977). An *in vitro* study shows that MMPs, particularly MMP-2 and MMP-9, are involved in the gingival ECM degradation during periodontitis (Ejeil*et al*., 2003). Therefore, it is interesting to investigate the expression and regulation of MMP-2 and MMP-9,

produced by local cells of periodontal tissues, including human gingival fibroblasts and human gingival epithelial cells.

2.4 Matrix metalloproteinases (MMPs)

MMPs are a family of zinc-containing endopeptidases,involved in remodeling and degradation of the ECM. To date, 25 membersof the MMP family have been identified and characterized, including 22 MMPs that are found in the human genome (Sorsa*et al*., 2006) (Table 2.1). MMPs generally consist of a prodomain, a catalyticdomain, a hinge region, and a hemopexin domain (Figure2.1). They are either secreted from the cell or anchored to theplasma membrane. MMPs are synthesized, secreted in a latent form (zymogens or proMMP), and activated by the proteolytic cleavage of their amino-terminal prodomain and the resulting conformational changes. The proteolytic activity of MMPs is regulated at multiple levels, including at the transcriptional level, the cleavage of their prodomain, the activity control by tissue inhibitors of matrix metalloproteinases (TIMPs), and the internalization by clearance (Nagase and Woessner, 1999; Westermarch and Kähäri, 1999; Uitto*et al*., 2003; Visse and Nagase, 2003; Sorsa*et al*., 2004; Folgueras*et al*., 2004).

Transcriptional activation can be stimulated by a variety of pro-inflammatory cytokines, hormones, and growth factors, such as, TNF-α, IL-1, IL-6, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. Activation of latent proMMPs can occur intracellularly, at the cell surface by membrane type (MT)-MMPs, and in the extracellular space by other proteases. Activated MMPs can further participate in the processing and activation of other MMPs in mutual activation cascades (Golub*et al*., 1994; Murphy and Knäuper, 1997; McCawley and

Matrisian, 2001; Egeblad and Werb, 2002; Visse and Nagase, 2003; Ala-aho and Kähäri, 2005; Björklund and Koivunen, 2005; Sorsa*et al*., 2006), leading to more activated enzymes that will result in more tissue destruction.

**Table 2.1 A family of human MMPs and their substrate specificities**

| Common name | MMP Number | Extracellular Matrix Substrates |
|---|---|---|
| Collagenases | | |
| – Collagenase-1 | MMP-1 | Collagens (I, II, III, VII, VIII and X) |
| – Collagenase-2 | MMP-8 | Collagens (I, II, III, V, VII, VIII and X) |
| – Collagenase 3 | MMP-13 | Collagens (I, II, III, IV, IX, X and XIV) |
| Gelatinases | | |
| – Gelatinase A | MMP-2 | Collagens (I, IV, V, VII and X), gelatin, elastin, fibronectin, aggrecan, laminin, decorin |
| – Gelatinase B | MMP-9 | Collagens (IV, V, VII and X), gelatin, elastin, fibronectin, aggrecan, fibronectin, proteoglycan link protein |
| Stromelysins | | |
| – Stromelysin 1 | MMP-3 | Collagens (III, IV, V and IX), gelatin, elastin, fibronectin, aggrecan, laminin |
| – Stromelysin 2 | MMP-10 | Collagens (III, IV and V), gelatin, aggrecan, laminin, proteoglycan link protein |
| – Stromelysin 3 | MMP-11 | Casein, laminin, fibronectin, gelatin, collagen IV |
| Matrilysins | | |
| – Matrilysin1 | MMP-7 | Collagens (IV and IX), gelatin, aggrecan, decorin, proteoglycan link protein |
| – Matrilysin2 | MMP-26 | Collagen IV, gelatin, fibronectin |

| | | |
|---|---|---|
| Membrane-type MMPs | | |
| a. Transmembrane | | |
| – MT1-MMP | MMP-14 | Pro-gelatinase A, collagens (I, II and III), elastin, fibronectin, gelatin, laminin |
| – MT2-MMP | MMP-15 | Pro-gelatinase A, large tenascin-C, fibronectin, laminin |
| – MT3-MMP | MMP-16 | Pro-gelatinase A, collagen III, gelatin, fibronectin |
| – MT5-MMP | MMP-24 | Pro-gelatinase A |
| b. GPI-anchored | | |
| – MT4-MMP | MMP-17 | Gelatin, pro-gelatinase A |
| – MT6-MMP | MMP-25 | Gelatin |
| Others | | |
| – Macrophage elastase | MMP-12 | Collagen IV, gelatin, elastin, laminin |
| – No trivial name | MMP-19 | Gelatin |
| – Enamelysin | MMP-20 | Amelogenin |
| – No trivial name | MMP-23 | ND |
| – Epilysin | MMP-28 | ND |

ND = not determined

(Modified from review articles by Steffensen*et al*., 2001; Visse and Nigase, 2003; Chakrabort*et al*., 2003)

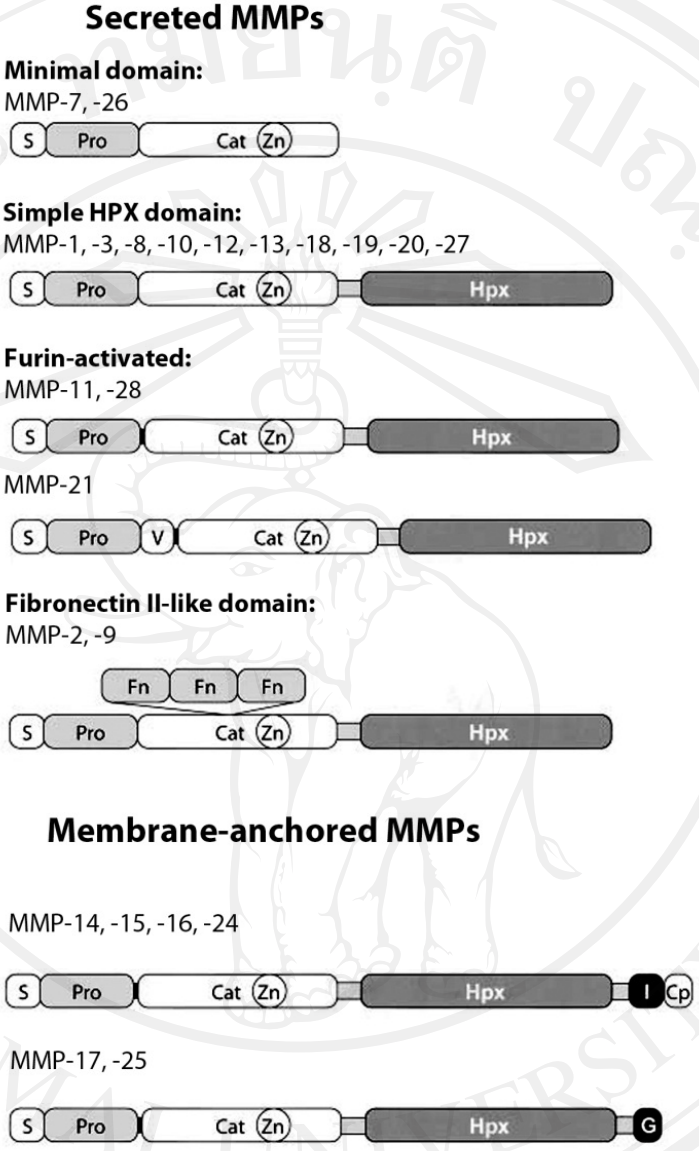


**Figure 2.1 The domain structure of MMPs.** The domain organization of MMPs is as indicated: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Zn, active-site zinc; Hpx, hemopexin domain; Fn, fibronectin domain; V, vitronectin insert; I, type I transmembrane domain; G, GPI anchor; andCp, cytoplasmic domain. A furin cleavage site is depicted as a black band between propeptide and catalytic domain. (Modified from Visse and Nigase 2003; Hadler-Olsen *et al*., 2011)

MMPs are controlled by endogenous inhibitors, TIMPs (Bauer *et al*., 1975), which are currently consisted of four known TIMPs, and they operate with different inhibition efficiencies against the different MMPs. The TIMPs are slow, tight-binding inhibitors with low molecular constants. The four TIMPs are differently expressed in tissues. TIMP-1 is localized in the bone and plays a more specific role in bone development. TIMP-2 is constitutively produced, as is MMP-2, with which it is normally paired. This pairing is in fact required for the on-demand activation of MMP-2 (Brassart*et al*., 1998). TIMP-3 is localized in the ECM, and TIMP-4 is localized mostly in vascular tissue.

Substrate specificity for the MMPs is not yet fully characterized. Known substrates include most of the ECM components (fibronectin, vitronectin, laminin, entactin, tenascin, aggrecan, myelin basic protein, etc.). The collagens (type I-X and XIV) have been shown to be substrates of different MMPs, with greatly different efficacies (Overall, 2001). In addition to connective tissue and ECM components, proteinase inhibitors, such as, $\alpha_1$-proteinase inhibitor, antithrombin-III and $\alpha_2$-macroglobulin, are selectively cleaved by MMPs. Intact type I collagen has been well studied as a substrate for some MMPs. MMP-1 cleaves intact triple helical collagen efficiently, but does not work well on other substrates. Other MMPs, such as, MMP-3 and MMP-7, cleave a broad range of substrates.

MMPs are involved in many physiologic and pathological processes, which include development and wound healing (Saloe*t al*., 1991; Mohan *et al*. 1998; McCawley*et al*., 2000), tooth eruption, postpartum uterus involution(Birkedal-Hansen, 1995), tumor invasion and metastasis (Wilhelm *et al*., 1989; Saloe*t al*., 1991; Sato and Seiki, 1993; Stähle-Bäckdahl and Parks, 1993; Kobayashi *et al*., 1996;

Coussens*et al*., 2000), bullous disease (Stähle-Bäckdahl*et al*., 1994; Verraes*et al*., 2001), angiogenesis (Nielsen *et al*., 1997; Berger*set al*., 2000), and apoptosis in oral lichen planus, which is found to be involved with MMP-9 (Zhou *et al*., 2001). The expression and activity of MMPs in adult tissue is normally quite low, but increases significantly in various pathological conditions that may lead to unwanted tissue destruction, such as, inflammatory diseases, tumor growth and metastasis (Sorsa*et al*., 2004). MMPs, therefore, have a marked role in tissue destruction of several oral diseases. Particularly, MMP-1, -2, -3, -8, and -9 have been found and their expression are increased in human biopsy specimens from inflammatory periodontal disease (Birkedal-Hansen, 1993; Reynolds, 1996; Westerlund*et al*., 1996). Furthermore, MMP-2 has been considered a major enzyme involved in the turnover of extracellular matrix (ECM) (Creemers*et al*., 1998).

MMP-2 (type IV collagenase; gelatinase A; 72 kDagelatinase)degrades denatured collagens, gelatins, as well as type IV collagen, a principle structural component of basement membrane (Aimes and Quigley, 1995). Most cells of mesenchymal origin have the ability to synthesize and secrete MMP-2 in pro-form (pro-enzyme), and the pro-form of MMP-2 requires activation for its catalytic activity, possibly by cleavage of the N-terminal prodomain. MMP-2 is secreted mainly by fibroblasts, predominant cells in connective tissue.

A study using *in situ* hybridization and immunohistochemistry has demonstrated that MMP-2 is expressed in healthy gingiva (Tervahartiala*et al*., 2000). This is confirmed by the result from tissue extracts, which demonstrated that the latent form of MMP-2 is expressed in all samples examined, while the active form of MMP-2 is detected only in tissue obtained from patients with clinical disease (Korostoff*et al*., 2000).

Furthermore, a study in human gingival tissue shows a significantly higher MMP-2 expression, which is detected by immunohistochemistry, in the untreated chronic periodontitis groups compared with the healthy or the gingivitis group (Dong *et al*., 2009).

MMP-2 is constitutively expressed by many cell types (Brown *et al*., 1996). MMP-2 function is controlled at three levels; transcription, activation, and inhibition. MMP-2 activation is different from that of other MMPs, since MMP-2 activation requires the formation of a trimolecular complex, consisting of MT1-MMP, TIMP-2, and MMP-2 on the cell surface (Stronginet *al*., 1995) (Figure 2.2). The MT1-MMP mechanism of proMMP-2 activation has been well studied and partially defined. The suggested model implicates TIMP-2 as a bridging molecule, tethering proMMP-2 through binding between the COOH-terminal ends of proMMP-2 and TIMP-2, and binding between the $NH_2$-terminal ends of MT1-MMP and TIMP-2. The propeptide of proMMP-2 is then cleaved by an adjacent TIMP-2-free MT1-MMP, generating an active intermediate form that will be further processed to the fully activated form of MMP-2 (Sato and Takino, 2010).Some studies have shown that the plasma membrane fraction from cultured cells can activate proMMP-2 (Azzam and Thompson, 1992).

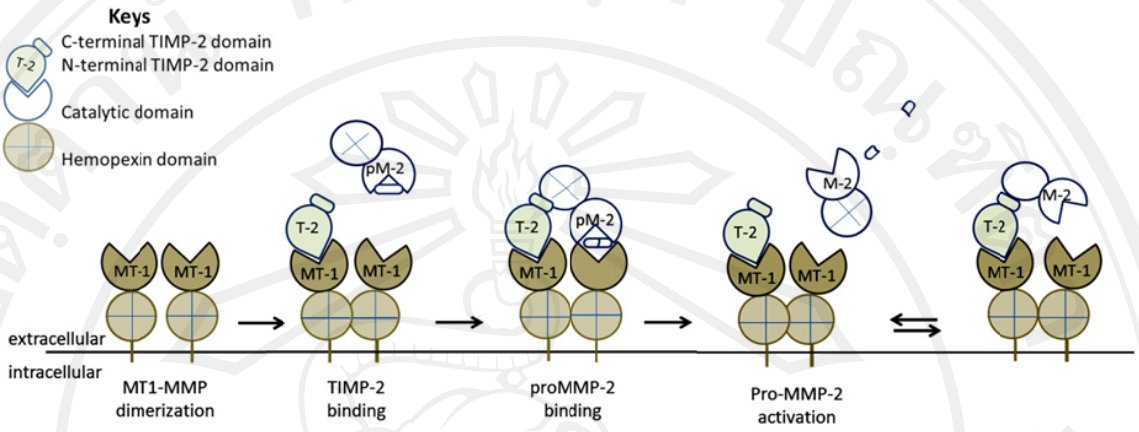


**Figure 2.2 A model of proMMP-2 activation by MT1-MMP and TIMP-2.** Active MT1-MMP (MT-1) on the membrane binds a molecule of TIMP-2 (T-2), inhibiting its activity. MT1-MMP can form dimers or multimers on the cell surface through interaction of itshemopexin domains. ProMMP-2 (pM-2) subsequently binds to the C-terminal domain of TIMP-2 through its hemopexin domain. The second active MT1-MMP then cleaves the bait region of proMMP-2, thereby partly activating it. The MMP-2 (M-2) dissociates from the membrane and is fully activated by intermolecular processing. (Modified from Visse and Nigase 2003)

MMP-9, also known as gelatinase B, has been suggested to play many roles in tissue metabolism of many pathophysiological conditions. Immune cells are well known to express MMP-9. However, a recent study has shown that local resident cells, like keratinocytes, can express MMP-9, while fibroblasts only express MMP-2 (Sawicki*et al*., 2005). The primary substrate for MMP-9 is first reported tobe gelatin, which is the remaining denatured collagen afterthe proteolytic cleavage of type I, II, or III collagen molecules by MMP-1, MMP-8, or MMP-13, yet numerous other substrates can also be cleaved by MMP-9 *in vitro* (Matrisian, 1990; Birkedal-Hansen, 1995).

In intact normal tissues, the expression of MMP-9 is maintained at detectable low and quiescent levels (Smith *et al*., 2004). In contrast, the activity and expression of MMP-9 in periodontal disease have been shown to elevate with increasing disease severity (Westerlund*et al*., 1996) and to decrease following periodontal treatment (scaling and root planing) (Golub*et al*., 1994; Sorsa*et al*., 2004; Uitto*et al*., 2003). Although several *in vitro* and clinical studies support the association between high levels of MMP-9 expression and the activity and severity of periodontal disease, little is still known about the up-regulation of MMP-9 in response to periodontal microorganisms,particularly in primary human gingival epithelial cells. Therefore, it is interesting to investigate the biology of MMP-9 in terms of the up-regulation of its synthesis and secretion in human gingival epithelial cells that are challenged with *F.nucleatum*, a Gram-negative periodontal bacterium.

2.5 *Fusobacteriumnucleatum* (*F. nucleatum*)

*F.nucleatum* is a non-spore-forming, non-motile, and Gram-negative anaerobic bacterium. *F. nucleatum* is commonly found in periodontal disease and can produce various tissue irritants, such as, butyric acid, proteases, and cytokines. It has strong adhesive properties due to the presence of lectins, and these outer membrane proteins mediate adhesion to epithelial and tooth surfaces, and co-agglutination with other suspected pathogens (Roberts, 2000). Moreover, *F.nucleatum* is one of the species that is significantly related to periodontal disease, and it is one of the most common anaerobes present in clinical infections, such as, sinusitis, pelvic infections, osteomyelitis, and brain and lung abscesses (Dzink*et al*., 1985; Feuille*et al*., 1996). *F.nucleatum* constitutes considerable part of the subgingivalmicrobiota of gingivitis in children and adults and of periodontitis in juveniles and adults (Kononen*et al*., 1994). The role of *F.nucleatum* in the development of periodontal disease has lately attracted new interest. Of over 51,000 clinical isolates examined by Moore and Moore (1994), *F.nucleatum* and *Actinomycesnaeslundii* are highly prevalent during the early stages of inflammation associated with gingivitis. From the early to the late stages of plaque formation, there is a shift from Gram-positive to Gram-negative microflora, in which *F.nucleatum* increases in proportion as dental plaque biofilm matures (Bolstad*et al*., 1996).

From studies on the bacteriology of experimental gingivitis in children (4 to 6 years old) and young adults (22 to 31 years old), *F.nucleatum* appears to be one of the non-spirochetal microorganisms most closely correlated with gingivitis, and it appears to be more common in young adults (Moore *et al*., 1982; 1984). This also seems to be the case in naturally occurring gingivitis (Isogai*et al*., 1985; Moore *et al*., 1987).

*F.nucleatum* is known to possess some virulence factors that can be classified as a periodontal pathogen. One important feature is the production of toxic metabolites, such as, butyrate, propionate, ammonium ions, etc., which can penetrate the gingival epithelium and are present in elevated levels in plaque biofilm associated with periodontitis. Comparison of direct cytotoxicity of sonicate extracts from several periodontal bacteria on human gingival fibroblasts has shown that the sonicate extracts from *F.nucleatum* and *A.actinomycetemcomitans* consistently appear to have the most profound cytotoxic effects at the lowest concentrations tested (Stevens and Hammond, 1988).

The ability of these microbial extracts to kill or arrest the proliferation of the normal periodontal resident cells, *i.e.*, human gingival fibroblasts, indicates that *F.nucleatum* plays an etiological role in periodontal disease as well (Singer and Buckner, 1981; Lopatin*et al*., 1985; Lopatin and Blackbur, 1986; Stevens and Hammond, 1988; Bartold*et al*., 1991). Although it seems that the effect of these metabolites is not toxic to the point of causing cell death, the inhibition of fibroblast proliferation may be severe enough because the potential for rapid wound healing is compromised.

In addition to the production of toxic metabolites, *F. nucleatum* has an ability to synthesize sulfide. The formation of sulfide may provide a way for *F. nucleatum* to escape important parts of the host immune system, since it is probable that the sulfide interferes with bacterial opsonization (Granlund-Edstedt*et al*., 1993). Furthermore, *F. nucleatum* has other abilities to adhere to type IV collagen and to degrade basement membrane *in vivo* (Winkler *et al*., 1987; Xie*et al*., 1991) and binds to and invades both gingival epithelial and endothelial cells, inducing host inflammatory responses

(Han *et al*., 2000). *F. nucleatum* has synergistic effects when acting together with other periodontopathogenic microorganisms, and possesses major outer membrane proteins that are important for its virulence (Bolstad*et al*., 1996; Feuille*et al*., 1996).

In this study, the cell wall extract of *F. nucleatum* strain ATCC25866 were used to stimulate human gingival fibroblasts and human gingival epithelial cells in an *in vitro* culture model. This is because it has previously been shown that the membrane components in the cell wall extract of *F. nucleatum* can induce expression of several effector molecules in innate immune response, such as, TNF-α, IL-8, human β-defensin-2 (hBD-2), an antimicrobial peptide of the β-defensin family (Krisanaprakornkit*et al*., 2000).

Consequently, the use of the cell wall extract of *F. nucleatum* to induce MMP-2 expression in gingival fibroblasts and MMP-9 expression in gingival epithelial cells is logical, since MMP-2 and MMP-9 are also classified as the effector molecules in innate immune response. In addition, the use of *F. nucleatum* cell wall extract as one of the cell activators in this proposal is rather convenient in that a large batch of *F. nucleatum* cell wall extract can be prepared at the beginning of the study and used throughout the whole project. This large amount of *F. nucleatum* cell wall extract also makes the findings obtained from all proposed experiments in this proposal more consistent and reproducible as variations between different lots ofbacterial cultures can be avoided.

2.6 Phospholipase D (PLD)

Phospholipase D (PLD), or called a phospholipid phosphohydrolase, hydrolyzes phosphatidylcholine (PC), a major component of phospholipid membrane, to yield

phosphatidic acid (PA), an intracellular signaling lipid mediator, and free choline (Heller, 1978; Billah and Anthes, 1990). PLD, ubiquitously present in all mammalian cells and tissues, is recognized as a receptor-regulated signaling enzyme that modulates many cellular functions (Cummings *et al*., 2002). Moreover, the PLD activity can be found in a variety of organisms, such as, plants, viruses, bacteria, fungi, mammals (Jenkins and Frohman, 2005).

PLD can catalyze transphosphatidylation reaction using water or primary alcohols, including ethanol and 1-butanol, to generate PA, or phosphatidylethanol (PEt) and phosphatidylbutanol (PBut), respectively (Figure 2.3). This reaction has been used as an assay to quantify the PLD activity. Accumulated evidence has indicated that PA, one of the products from the PLD activity, plays an essential role in the regulation of various important biological events, including motility, cytoskeletal remodeling, proliferation, membrane trafficking. Furthermore, PA can act as a substrate for several specialized products, such as, lysophosphatidic acid (LPA), etc., which have been linked to several biological processes, including vesicular traffic, secretion, and endocytosis (Jenkins and Frohman, 2005).

In mammals, the PLD activity is found in most cell types with the exception for leukocytes and a few lymphocyte lines (Jenkins and Frohman, 2005). To date, two mammalian PLD isoforms, *i.e.*, PLD1 and PLD2, have been identified (Hammond *et al*., 1995; 1997; Colley *et al*., 1997; Steed *et al*., 1998). Human PLD1, a 120 kDa protein, has two splice variants, *i.e.*, PLD1α and PLD1β(Hammond *et al*., 1997; Katayama *et al*., 1998) that show similar regulatory properties and regions, which are both stimulated with phosphatidylinositol 4,5-bisphosphate ($PIP_2$), phosphatidylinositol 3,4,5-triphosphate ($PIP_3$), $Ca^{2+}$, phorbol ester, protein kinase C

(PKC), the Rho family of small GTPase, and ADP ribosylation factor (ARF) GTPase proteins (Katayama *et al*., 1998; Hammond *et al*., 1995; Kodaki and Yamashita, 1997). However, PLD2 exhibits a significant higher basal activity in the presence of $PIP_2$ (Colley *et al*., 1997), but it is not activated by PKC, Rho GTPase (Lopez *et al*., 1998), or ARF (Colley *et al*., 1997).

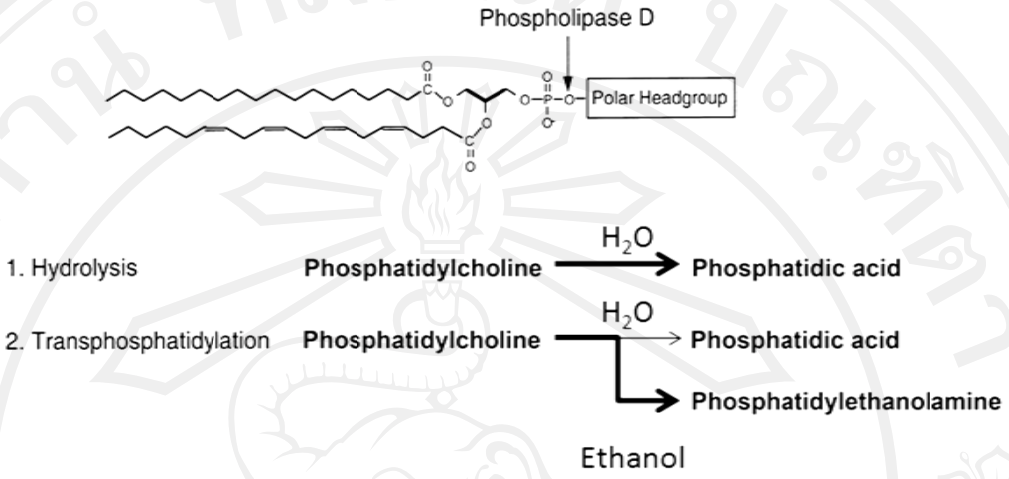


**Figure 2.3 Phospholipase D-catalyzed reactions.** Phospholipase D hydrolyzes the distal phosphodiester bond in phospholipids. A phosphatidyl-enzyme intermediate is normally subject to nucleophilic attack by water. However, primary short chain alcohols (*e.g.*, methanol, ethanol, 1-propanol, 1-butanol) can substitute for water in a competing transphosphatidylation reaction. The product of PLD-catalyzed transphosphatidylation is the corresponding phosphatidic acid alkyl ester, or phosphatidyl-alcohol. (Modified from Liscovitch*et al*., 1999)



There have been several studies that show the subcellular localization of PLD1 and PLD2 in numerous cell lines. The localization of PLD1 has been described as

perinuclear at the Golgi, endoplasmic reticulum, and late endosomal distribution (Colley *et al*., 1997; Sung *et al*., 1999; Stahelin*et al*., 2004; Freyberg *et al*., 2001). However, PLD1 translocates to plasma membrane upon stimulation (Du *et al*., 2003; Brown *et al*., 1998; Huang *et al*., 2005). PLD2 is most often reported to localize at the plasma membrane (Colley *et al*., 1997; Honda *et al*., 1999; Du *et al*., 2003), cytosol (Honda *et al*., 1999), and sub-membranous vesicular compartment (Divecha*et al*., 2000).

Consistent with the proposed regulatory role of PLD1 in MMP-9 expression and secretion in this study, a few studies have shown that the secretion of MMP-9 is stimulated with the introduction of PA into the growth medium, that inhibition of PA production by 1-propanol inhibits MMP-9 secretion, and that the addition of dioctanoylphosphatidic acid (DOPA) also induces MMP-9secretion in HT 1080 fibrosarcoma cells (Willige*et al*., 1999; Ho *et al*., 2003). These results suggest a PA-dependent mechanism in MMP-9 secretion and imply a role of PLD in regulating MMP-9 secretion. However, to my knowledge, there has been no study regarding the regulation of MMP-9 expression and secretion by PLD in primary epithelial cells. Therefore, it is interesting to investigate a role of PLD in MMP-9 expression and secretion in gingival epithelial cells, which has previously been found to be involved in up-regulating human β-defensin-2 expression upon stimulation with the cell wall extract of *F. nucleatum* (Krisanaprakornkit*et al*., 2008).



2.7 Phospholipase $A_2$($PLA_2$)

$PLA_2$ is a family of enzymes that liberate free fatty acids from the *sn*-2 position of glycerophospholipids to produce free fatty acids, including arachidonic acid (AA) and lysophospholipid (Figure 2.4). Both products serve as signaling molecules that can exert a multitude of biological functions (Dennis, 1994; 1997). AA may act as an intracellularsecond messenger or can be converted into eicosanoids through the action of a variety of prostaglandin synthases, lipoxygenases, and cytochrome P450 proteins (Funk, 2001).

Eicosanoids play a role in a wide range of physiologic and pathological processes, such as, sleep regulation, immune responses, inflammation, and pain perception. Eicosanoids act through binding to specific G protein-coupled receptors (Tsuboi*et al*., 2002). The lysophospholipid can serve as a precursor for lipid mediators, such as, LPA, platelet activating factor (PAF), etc. LPA is involved in cell proliferation, survival, and migration (Moolenaar*et al*., 2004), whereas PAF is particularly involved in inflammatory processes (Prescott *et al*., 2000). Similar to the receptors for eicosanoids, LPA and PAF also act through binding to G protein-coupled receptors.

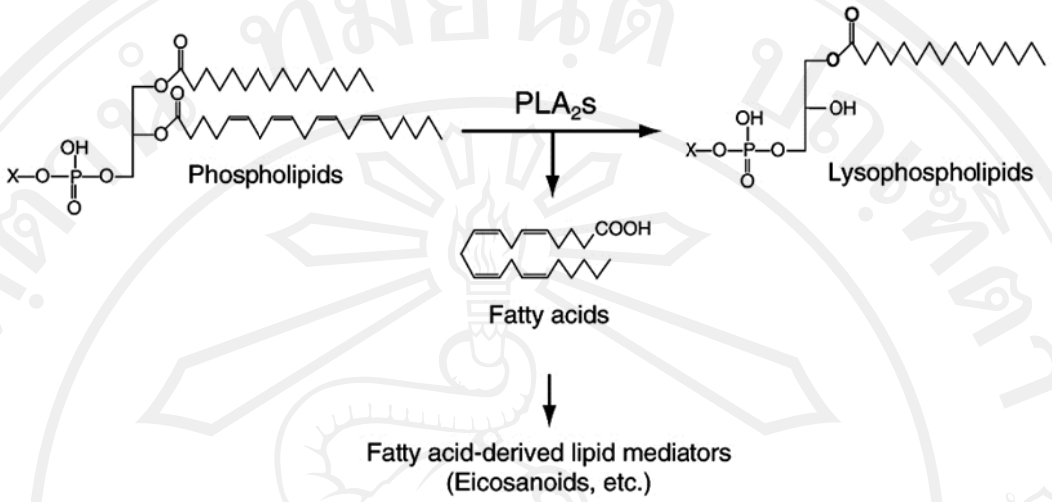


**Figure 2.4Phospholipase $A_2$ on the metabolic pathway of lipid mediator production.** $PLA_2$ hydrolyzes membrane glycerophospholipids to generate fatty acid metabolites and lysophospholipids, both of which can be biologically active lipid mediators. Cytosolic $PLA_2\alpha$ prefers arachidonic acid-containing glycerophospholipids as a substrate, playing a critical central role in inflammatory eicosanoids. (Modified from Kita *et al*., 2006)

$PLA_2$ enzymes have been found in many cell typesand tissues. Based on their cellular localization, theyare classified into two general forms: extracellular (secreted)and intracellular (cytosolic)(Dennis, 1994; 1997). Generally, the secreted $PLA_2$s ($sPLA_2$s) have lower molecular masses between14 and 16 kDa, require higher calcium concentrationsfor their catalytic activity, and possess 5 to 7 disulfidebonds. The intracellular (cytosolic) $PLA_2$s ($cPLA_2$s)are further divided intoGroup IV and VI, based on the $Ca^{2+}$ requirementsneeded for their basal catalytic activity.While the $sPLA_2$s require millimolar calcium concentrations, the calcium-sensitive $cPLA_2$ only requires micromolar, or even sub-micromolar, calcium concentrations for its enzymatic activity.

Most studies have shown that $cPLA_2$ generally plays a regulatory role in gene expression in eukaryotic cells, whereas $sPLA_2$provides most of the AA release that occurs in response to receptor-agonist binding.Both $cPLA_2$and $sPLA_2$s have beenimplicated in various physiologic and pathological functions, including lipid digestion, release of pro-inflammatory mediators,cell proliferation, ischemic injury, inflammatory disease, cancer, and anti-bacterial defense (Bonventre, 1999; Valentin*et al*., 1999; Sapirsteinand Bonventre, 2000).

The Group IV and VI $cPLA_2$s have much larger molecular masses about 80–85 kDa, and areinsensitive to thiol-reducing agents, such as,dithiothreitol (DTT).In the Group IV $cPLA_2$, there are at least fourparalogsin mammals, including $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$ (Hirabayashi*et al*., 2004). The primary structure of each paralog in the Group IV $cPLA_2$ is shown in Figure 2.5. All of them contain two homologous catalytic domains A and B interspaced with gene-unique sequences (Figure 2.5). The lipase consensus sequence, GXSGS, is located in the N-terminal

region of the catalytic domain A. cPLA$_2$α, -β, -δ have an N-terminal C2 domain, which is involved in $Ca^{2+}$-dependent phospholipid binding (Figure 2.5) (Hirabayashi*et al*., 2004).

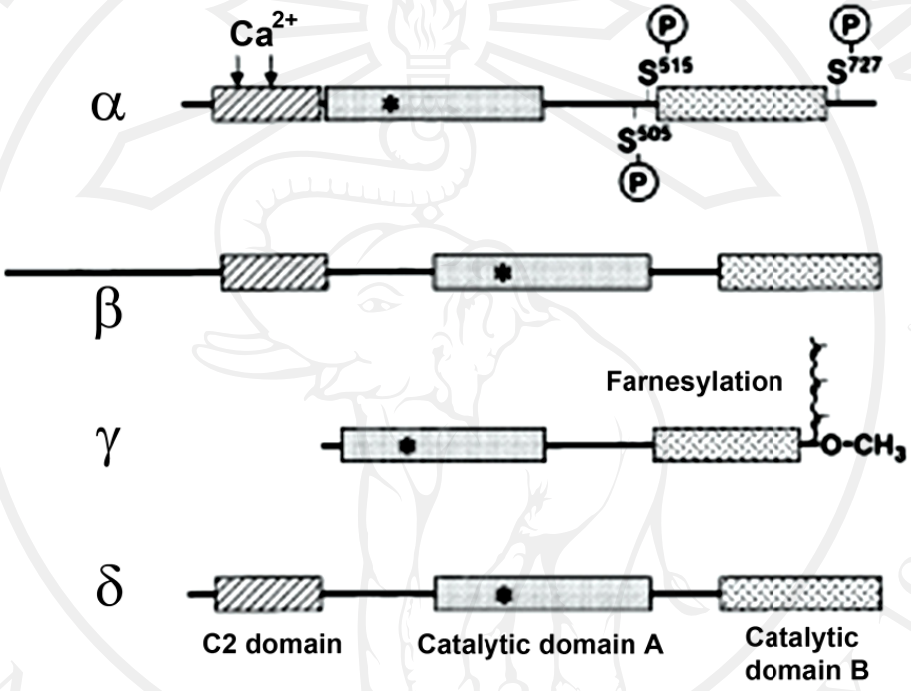


**Figure 2.5 The four paralogs of the Group IV cPLA$_2$.** This figure shows three conserved domains, including two catalytic domains, A and B, and the C2 domain, and posttranslational modifications by phosphorylation at different serine residues (S) and by farnesylation. An asterisk in the catalytic domain A of each paralog represents the location of the lipase consensus sequence. (Modified from Hirabayashi*et al*., 2004)

With respect to the role of $PLA_2$ in MMP-9 expression, it has been previously demonstrated that activation of monocytes/macrophages by pro-inflammatory stimuli, such as, lipopolysaccharide(LPS), concanavalin A (Con A), or lymphokines, leads to increased AA production, which in turn causes an elevation of $PGE_2$ and cyclic adenosine monophosphate(cAMP) that are required for the induction of MMP-9 synthesis in monocytes (Wahl *et al*., 1974; Wahl*et al*., 1975; Wahl and Lampel, 1987; Cury*et al*., 1988).Although LPS-mediated MMP-9 production in monocytes hasalready been known to involve the up-regulationof $PGE_2$ and cAMP levels, the early biochemical events that are responsible for MMP-9induction arenot well understood.

It is not until one study that shows the importance of tyrosine phosphorylation and the involvement of $cPLA_2$duringthe early stage of the signal transduction pathway forMMP-9 induction (Figure 2.6) (Shankavaram*et al*., 1998). This study provides evidence that tyrosine phosphorylation of $cPLA_2$ is among the initial step, needed for the LPS-induced MMP-9 production in human monocytes (Figure 2.6).The activated $cPLA_2$ by phosphorylation upon LPS stimulation leads to an increased production of AA, which serves as a substrate for cyclooxygenases and prostaglandin synthases to generate $PGE_2$ (Figure 2.6). Subsequently, the released $PGE_2$ can bind to its preferred receptor, *i.e.*,EP4 (Yan *et al*., 2005), which activates the production of cAMP (Figure 2.6). The increased intracellular cAMP levels will then enhance MMP-9 production in monocytes (Figure 2.6). Therefore, similar to human monocytes, it is probable that activation of $cPLA_2$ by phosphorylation also plays a crucial role in the early stage of the signal transduction pathway for MMP-9 up-regulation in human gingival epithelial cells.

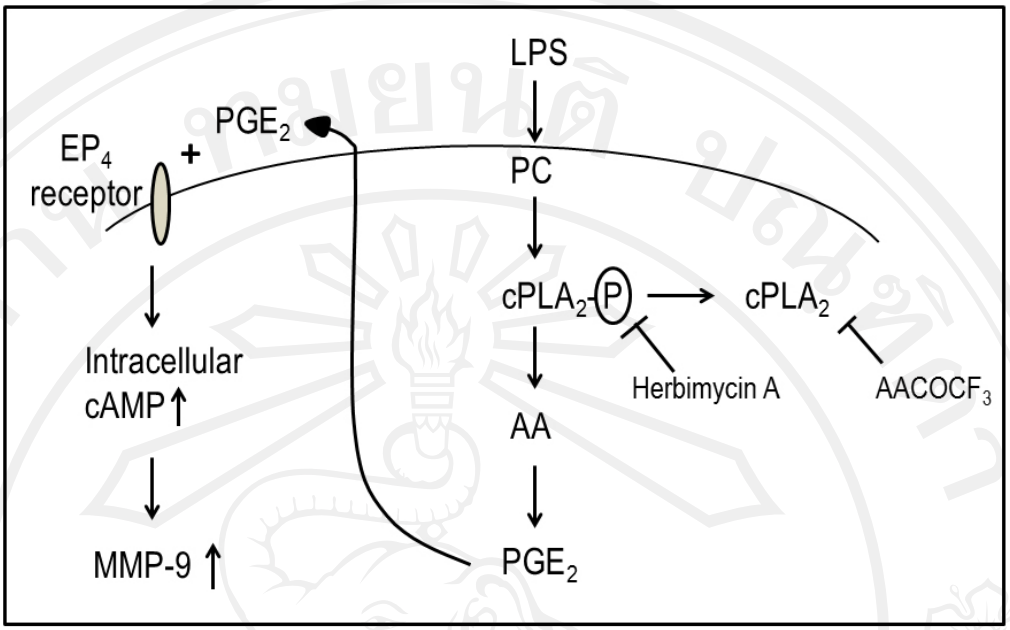


**Figure 2.6 A schematic diagram shows the importance of tyrosine phosphorylation and the involvement of $cPLA_2$ in MMP-9 up-regulation in human monocyte** (Shankavaram*et al.*, 1998). LPS stimulated-human peripheral blood monocytesare demonstrated to involve the phosphorylation of cytosolic phospholipase$A_2$($cPLA_2$) to generate arachidonic acid (AA) and prostaglandin $E_2$ ($PGE_2$), resulting in MMP-9 up-regulation via the binding between released $PGE_2$ and its cognate receptor, EP4, which then results in elevated levels of cyclic AMP (cAMP). Pretreatment with herbimycin A, a tyrosine kinaseinhibitor, or with arachidonyltrifluoromethyl ketone($AACOCF_3$), a specific inhibitor of $cPLA_2$, inhibits the induction of MMP-9 by LPS in human monocytesvia the $PGE_2$-cAMP dependent pathway.