CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Periodontal disease is chronic inflammation of tooth supporting structures including gingiva, connective tissue attachment and alveolar bone which caused by a complex interaction between host defenses and microorganisms in dental plaque (Sosroseno and Herminajeng, 1995). Periodontal disease could be clinically defined into two distinct groups, gingivitis and periodontitis (Caton et al., 1989). Initially, gingivitis represents a generalized acute inflammatory response to the bacteria that colonize on the tooth surface adjacent to the gingiva. With the time, gingivitis may become well established, but remain confined to the superficial gingival connective tissue and manifest all of the classic features of a chronic inflammatory lesion. If the inflammatory response contained within the gingivitis lesion spreads to the deeper periodontal tissues and alveolar bone is lost, the resultant lesion is called periodontitis. The precise mechanisms governing the progression of gingivitis to periodontitis are unclear. In some cases, gingivitis may represent the early stage in the evolution of periodontitis. However, in some individual, gingivitis may exist as an independent clinical condition without progressing into periodontitis (Williams, 1990). Such variability can be attributed to differences in composition of the microbial flora, together with the presence of factors that might modify the host response to microbial assault, as well as factors that may predispose the individual to bacterial colonization at specific sites. Of these, it seems that the microflora composition and the host modifying factors are the most important regarding manifestation of periodontal

disease. While the host response and environmental factors that affect this response are important for disease manifestation, gingivitis and periodontitis cannot commence without the presence of bacteria. Nevertheless, it must be noted that although bacteria are necessary for disease initiation, they are not sufficient to cause disease progression unless there is an associated inflammatory response. The latter overrides its protective role and permits destruction to occur (Offenbacher, 1996; Page and Beck, 1997).

A large number of bacterial species, nearly 500 species, colonize in oral cavity (Paster et al., 2001). However, approximately 20 microbes that inhabit the subgingival dental plaque are considered to be associated with periodontitis. The most significant bacteria associated with periodontitis are *Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis),* and *Bacteroides forsythus (B. forsythus)* (Zambon, 1996). An important emerging concept with respect to the subgingival microflora is that it behaves as a biofilm that permits the occupants to survive as a community and resist common host defense mechanisms as well (Darveau et al., 2000).

1.2 Pathogenesis of periodontal tissue destruction

1.2.1 Pathogenesis of gingivitis

Chronic marginal gingivitis is characterized clinically by gingival redness, edema, bleeding, changes in contour, loss of tissue adaptation to the teeth, and increase flow of gingival crevicular fluid (GCF) (Greenstein, 1984). Development of gingivitis requires the presence of plaque bacteria (Theilade et al., 1966) which are

though to induce pathological changes in the tissue by both direct and indirect pathways. Histopathologic observations have led to the subdivision of gingivitis into 3 stages (Page, 1986). The initial lesion appears as an acute inflammatory response with characteristic infiltration with neutrophils. Vascular changes, epithelial cell changes, and collagen degradation are apparent. These initial changes are likely due to chemotactic attraction of neutrophils by bacterial constituents and direct vasodilatory effects of bacterial products, as well as activation of host response such as complement and kinin systems and arachidonic acid (Attstrom and Egelberg, 1970; Payne et al., 1975). The early lesion is characterization by a lymphoid cell infiltrate dominate by T lymphocytes, with extension of collagen loss, while the establish lesion is dominate by B lymphocytes and plasma cells. Although direct evidence for specific mechanisms explaining the appearance and progression of gingivitis lesions is not available. The chronic inflammatory infiltrate characteristic of the early and established lesion, as well as the proliferation of the junctional epithelium and destruction of collagen, are consistent with the activation of mononuclear phagocytes and fibroblasts by bacterial products with the recruitment and activation of the local immune system and cytokine pathways. The progression of the lesion from acute inflammation through T cell and then B cell predominance is likely orchestrated by a progression of cytokines which are responsible for recruitment, differentiation, and growth of the characteristic cell types with progressive chronic lesion. Importantly, completely removal of plaque will usually result in resolution of the chronic gingivitis lesion without residual tissue destruction.

1.2.2 Pathogenesis of periodontitis

Periodontitis is clinically differentiated from gingivitis by the loss of connective tissue attachment to the teeth in the presence of concurrent gingiva inflammation (Listgarten, 1986). Loss of periodontal ligament and disruption of its attachment to cementum, as well as resorption of alveolar bone occur. Together with loss of attachment, there is migration of epithelial attachment along the root surface and resorption bone (Page et al., 1980). The histopathology of periodontitis lesion is in many ways similar to that of the established lesion of gingivitis, with a predominance of plasma cells, loss of soft connective tissue elements and bone resorption. Despite the histopathologic similarity between gingivitis and periodontitis, evidence is lacking that would indicate that periodontitis is an inevitable consequence of gingivitis. (Socransky et al., 1984; Jeffcoat and Reddy, 1991; Reddy and Jeffcoat, 1993). Understanding the pathologic mechanism involved still awaits measurement methods that clearly differentiate between active and quiescent disease.

1.3 Microbial aspects of periodontal disease

For many years, it has been recognized that periodontal diseases are cause by accumulation of bacteria on the surface of the tooth and beneath the gingiva (Socransky and Haffajee, 1992). A healthy and gingivitis sites, composition of microbial plaque is quite similar and the majority is gram-positive, such as *Streptococci* and *Actinomyces* (Slots, 1977). In contrast, plaque associated with periodontitis appears to be specific group of bacteria or critical pathogens in mixed infection. Gram-negative rod anaerobes and also spirochete species are often found in

subgingival plaque of periodontitis patients (Dzink et al., 1988; Simonson et al., 1988).

It is widely accepted that the initiation and progression of periodontitis are dependent upon the presence of microorganisms capable of causing diseases. A large number of bacterial species, nearly 500 species, colonize in oral cavity (Paster et al., 2001). However, approximately 20 microbes that inhabit the subgingival dental plaque are considered to be associated with periodontitis. Most of all are gram-negative, non-motile rod and also spirochete species. At least 3 characteristics of periodontal microorganism have been identified that can contribute to their ability to act as pathogens. The capability to colonize, the ability to evade antibacterial host defense mechanisms and the ability to produce substances that can directly initiate tissue destruction. The most significant bacteria associated with periodontitis are *A. actinomycetemcomitans, P. gingivalis* and *B. forsythus* (Zambon, 1996).

1.4 Pathogenic strategies of *Porphyromonas gingivalis*

In general, severe forms of periodontal disease in adults are associated with a number of gram-negative anaerobes. Of these groups, most evidence points to a pathogenic role for *Porphyromonas* (formerly *Bacteroides*) *gingivalis.* The presence of this organism, acting either alone or as mixed infection with other bacteria, and possibly in concert with the absence of beneficial species and certain immunological deficiency in host, appears to be essential for disease activity. Intensive study of *P. gingivalis* has revealed a vast array of potential virulence factors that are now being defined at a molecular level (Sojar et al., 1997; McGraw et al., 1999).

1.4.1 Entry to oral cavity

Initial entry of *P. gingivalis* into the oral cavity is thought to occur by transmission from infected individuals (Greenstein and Lamster, 1997). Saliva is considered an important vector for transmission. The clonal diversity of *P. gingivalis* isolates has been examined by variety of techniques including ribotyping, restriction endonuclease analysis, restriction fragment length polymorphism, multilocus enzyme electrophoresis, and arbitrary PCR amplification (Loos et al., 1990; Loos et al., 1993; Menard and Mouton, 1995; Teanpaisan et al., 1996). These studies indicate that individuals are colonized by a single genotype, regardless of site of colonization or clinical status. Strains of many different clonal origins are present in different individuals. This supports the concept that *P. gingivalis* is essentially an opportunistic pathogen, with virulence not being restricted to a particular clonal type.

1.4.2 Adherence to oral surface and adhesin molecules

The oral cavity provides a variety of surfaces to which *P. gingivalis* can adhere. The adherence could be found not only the mineralized hard tissues of the teeth but also the mucosal surfaces including gingiva, cheek, and tongue. The microbial coating of freshly cleaned tooth surface occurs rapidly. Within the first few hours a pellicle forms on the tooth surface that consists of protein and glycoproteins found in saliva and crevicular fluid (Marsh and Bradshaw, 1995). After pellicle formation, the surfaces provide an appropriate bacterial attachment and followed by an additional bacterial colonization (Skopek and Liljemark, 1994). The first colonizer such as *Streptococcus* species and *Fusobacterium* are the most predominant pellicle

colonizers and provide an abundant array of bacterial adhesions after attachment. They are therefore proposed to play a major role in biofilm formation (Whittaker et al., 1996). *Fusobacterium* species could directly coaggregate with other species and also *P. gingivalis* as well. Coaggregation is based on the specific interaction of proteinaceous adhesion produced by one bacterium and a respective carbohydrate or protein receptor found on the surface of another bacterium. Adhesin can be anchored to the cell wall or membrane, and receptors are generally cell wall polysaccharides that contain strain-specific saccharide repeating units (Page and Kornman, 2000).

Ultrastructural examination on *P. gingivalis* outer membrane has revealed the presence of peritrichious fimbriae, 0.3-3.0 μm long and 5 nm wide. Fimbriae are composed of fimbrillin monomer subunit that varies in size between 41-49 kDa. The carboxy-terminal region of the fimbrillin subunit of fimbriae appears to be responsible for binding to surface-bound salivary components. Furthermore, fimbria associated protein may also be involved in adherence to epithelial cells (Lamont et al., 1995; Weinberg et al., 1997). The gene encoding the fimbrillin subunit (*fimA*) has been cloned, and there is considerable heterogeneity in this gene between *P. gingivalis* strains; these differences may be important in the function and immunogenicity of the fimbriae (Loos and Dyer, 1992). Immunization with purified native fimbriae has been shown to protect against periodontal tissue destruction in the gnotobiotic rat model (Evans et al., 1992). In addition, a *P. gingivalis fimA* mutant did not induce bone loss in this model (Malek et al., 1994). Similarly, recent study has demonstrated that a *P. gingivalis fimA* mutant do not adheres as well to tissue-culture human gingival fibroblasts and epithelial cell. Taken together, these studies indicated that *P. gingivalis* fimbriae are important in virulence, possibly by mediate initial adherence

and colonization of oral cavity, which may be the first step in initiation of periodontal tissue destruction (Hamada et al., 1994). This apparatus appears to be a major adherence-mediating determinant of *P. gingivalis.*

1.4.3 Ability of hemagglutination

Although fimbriae were thought to be largely responsible for the hemagglutinating activity of *P. gingivalis*, an outer-membrane protein, designated HA-Ag 2, that is distinct from fimbrillin and that has high binding affinity for human erythrocytes has since been identified (Mouton et al., 1991). Two distinct hemagglutinin-encoding genes, *hagA* and *hagB* have been identified in *P. gingivalis* strain 381 (Progulske-Fox et al., 1993). Hemagglutinin proteins are established virulence factors for a number of bacterial species, and *P. gingivalis* produce at least 5 hemagglutinin molecules. These protein promote colonization by mediating the binding of bacteria cell to erythrocytes, which may served a nutrition function and also to receptors (usually oligosaccharide) on human cells (Lepine and Progulske-Fox, 1996). *P. gingivalis* has invasive potential, actively internalizing within epithelial cells by mechanisms similar to those used by various enteric pathogen. This process induces changing in cell surface receptors or intracellular signaling pathway (Lamont et al., 1995).

1.4.4 Nutrient acquisition : Peptides and hemin

Peptides

Importantly, *P. gingivalis* is an asaccharolytic organism. Thus, their growth depends on the nutrient from nitrogenous substrate. Although sugars such as glucose can be utilized by the organism, these compounds are not converted to metabolic end products but, rather, are used for biosynthesis of intracellular macromolecules (Shah and Gharbia, 1989; Shah and Gharbia, 1993). Particularly dipeptides are efficiently utilized for growth (Takahashi and Sato, 2002). Thus, the action of proteolytic enzymes produced by *P. gingivalis*, along with other bacterially and host-derived proteinase, in the protein-rich subgingival milieu would appear to be pivotal to nutrient acquisition by *P. gingivalis.*

Several enzymes synthesized by *P. gingivalis,* including hyaluronidase, keratinase and superoxide dismutase, have been implicated in invasion, tissue destruction and evasion of oxygen dependent bactericidal activity of neutrophils (Lynch and Kuramitsu, 1999). Nevertheless, the primary focus of research has been on the proteinases, which are produced, in large quantities by this organism. These enzymes have been shown to evoke a diverse variety of potentially harmful activities, including (i) activation of the kallikrein/kinin cascade (Imamura et al., 1994), (ii) inactivation of host proteinase inhibitors (Carlsson et al., 1984a), (iii) dysregulation of the coagulation cascade (Nilsson et al., 1985); (iv) dysregulation of complement cascade (Schenkein and Berry, 1988; Sundqvist et al., 1988a), (v) degradation of immunoglobulins A1, A2, G and iron transporting protein (Kilian, 1981; Sundqvist et al., 1988b), (vi) degradation of cell surface protein and matrix glycoproteins (Uitto et

al., 1989) (vii) alteration of neutrophils antimicrobial activities (Yoneda et al., 1990; Lala et al., 1994); (viii) deregulation of inflammatory processes during the course of periodontal infection by cleavage of hormone, neuropeptide and proteolysis of cytokines (IL-1β) and salivary proline-rich peptide (Banbula et al., 2000).

Collectively, bacterial proteinase diversities of these make them immediately apparent that *P. gingivalis* can significantly contribute to virulence and could be responsible for most of clinical hallmarks of periodontitis progression.

Hemin

Hemin is one of the important requirements during their growth. *P. gingivalis* has an obligate iron requirement for growth. A number of hemin-containing compounds, such as hemoglobin, haptoglobin, myoglobin, hemopexin, methhemoglobin, oxyhemoglobin, albumin, lactoperoxidase, catalase, and cytochrom *c*, can provide hemin following proteolytic processing (Fujimura et al., 1995; Smalley et al., 1998; Bramanti and Holt, 1991; Banbula et al., 2001).

Under condition of hemin excess, *P. gingivalis* were heavily fimbrieated, which may aid their colonization to host surface, a long with this evidence they also produce a large amount of cytotoxic metabolic products such as butylrate. In addition, cells grown under hemin-limited condition appear as short rod, whereas those cultured under hemin-excess condition were coccus shaped (McKee et al., 1986). In contrast, *P. gingivalis* can produce extracellular vesicles by outgrowths of the outer membrane under certain growth condition (medium lacking hemin) These vesicles contain the same lipopolysaccharide (LPS) and array of enzymatic material s as parent cell. They

vary in size from 20-500 nm and also exhibit the same proteolytic activities as whole cell. (Guigand et al., 1995) Thus, a clinical symptom of bleeding from gingival sulcus was served as hemin-enrichment for their growth. This activity also regulated by hemolysin, which is responsible for erythrocyte lysis.

1.4.5 Potentially destructive compounds

Many studies reported that enzymes and metabolic products that could be detrimental to the host. These compounds include phospholipase A, which can provide the prostaglandin precursor that could stimulate prostaglandin-mediated bone resorption (Bulkacz et al., 1981); alkaline and acid phosphatase, which may contribute to alveolar bone breakdown (Frank and Voegel, 1978; Slots, 1981); DNase and Rnase (Rudek and Haque, 1976); sialidase (Holt and Bramanti, 1991); volatile sulfur compounds such as hydrogen sulfide, methylmercaptan, and dimethyl disulfide, which are cytotoxic and can inhibit protein synthesis (Tonzetich and McBride, 1981; Slots and Genco, 1984); butyrate and propionate, which are cytotoxic for epithelial cells, fibroblasts, and lymphocytes (Singer and Buckner, 1981; Slots and Genco, 1984; Kurita-Ochiai et al., 1997); and indole and ammonia, which also exhibit cytotoxicity (Slots and Genco, 1984; van Winkelhoff et al., 1988).

From these observations, *P. gingivalis* is well equipped with a wide array of structural and functional features, which enable it to colonize either the gingival sulcus or periodontal pockets. This organism could survive in this microenvironment of host by successfully evading host antimicrobial defenses. They also utilized such putative virulence factors as fimbriae and lectin-type adhesions, a polysaccharide

capsule and lipopolysaccharide, hemagglutinating and hemolysin, release of toxic products of metabolism, outer membrane vesicles and numerous enzymes (Mayrand and Holt, 1988; Imamura et al., 1995; Lamont and Jenkinson, 1998; Smalley and Birss, 1991). Eventhough other species may be involved in causing periodontitis, but *P. gingivalis* act more directly on periodontal destruction than other species. Therefore, *P. gingivalis* would appear to be studied for the understanding the mechanism of periodontal destruction to develop the effective prevention and treatment of periodontal disease.

Eventhough, bacteria are necessary for disease initiation but insufficient to cause the disease progression. The bacterial component was accounted for a relatively small proportion (approximately 20%) of the variance in disease expression. Host factors are more important than the bacteria in determining disease development and outcome. The complex interplay between the bacterial challenge and host factors determines the outcome.

1.5 Host response to plaque microorganism

Although the pathogenesis of periodontal disease is not fully understood, it is known that interaction of host defense mechanisms and etiologic agents is an important determinant of the onset and progression of the disease. There is well-documented evidence that bacteria and their products found in dental plaque comprise the primary etiologic agents responsible for periodontal disease (Genco et al., 1969; Riviere et al., 1995; Socransky et al., 1998). Base on an advanced knowledge in three specific areas of bacterial involvement in periodontal tissue destruction. First, the

subgingival microflora is highly organized into biofilm and manifests the characteristics of biofilms. The biofilms are defined as "matrix-enclosed bacteria populations adherent to each other and/or to surfaces or interfaces" (Costerton et al., 1994). The bacteria are largely protected from the host defenses and are highly resistant to chemotherapeutic agents. Physical disruption by scaling and root planing is an effective treatment. Second, major advance have been made at the cellular, molecular and genetic levels in understanding the pathways and mechanisms by which the bacteria present in these biofilms initiate and perpetuate the immunoinflammatory response that destroy the connective tissue in gingiva and periodontal ligament and alveolar bone. Third, although bacteria cause periodontitis and are essential for disease initiation, bacteria alone are insufficient. A susceptible host is required. Acquire and environmental risk factors such as tobacco smoking as well as genetically transmitted trait modify the shared pathway by which bacteria cause tissue destruction and they determine disease susceptibility, onset, progression, severity and outcome. This new way looking at pathogenesis of periodontitis opens entirely new approaches to prevent and managing the diseases.

1.6 Mechanism of alveolar bone destruction in periodontal disease

The cells of the periodontium and also other connective tissues are embedded in an extracellular matrix (ECM) that regulates their activities and functions. The connective tissue matrix is composed of several organic constituents including collagen, noncollagenous proteins, and proteoglycan. Among these, collagens are the principle structural components. The collagen molecule is a rigid, rod-like structure that resists stretching, and fibers made up of collagen have high tensile strength.

Therefore, this protein is an important structural component in tissues such as periodontal ligament and tendon in which mechanical forces need to be transmitted without loss. The structure and content of collagens are affected under variety of pathological conditions (Bartold and Narayanan, 1998).

The biochemical composition of the periodontal connective tissues during health and disease is determined by the extents of degradation and synthesis of matrix constituents (Reynolds and Meikle, 1997; Reynolds and Meikle, 2000). Degradation is normal features of the periodontal connective tissues, particularly collagens, have high turnover rate relative to other tissues. The major change in biochemical constituents of the connective tissue during gingivitis and periodontitis is the loss of collagen contents due to degradation.

Connective tissue destruction may result from directly degrade by bacterial proteinases or by localized host-derived enzymes. For enzyme-mediated damage by host cell; there are at least 4 pathways for degradation of extracellular matrix components in periodontal tissue destruction. A body of evidence suggests that matrix components may be dissolved by matrix metalloproteinases (MMP)-dependent or plasmin (Pln)-dependent cleavage reactions, and that larger fragments of may be disposed of by a phagocytic pathway by way of lysosomal proteinases. Mineralized matrices appear to be degraded by a complex extra/ pericellular process mediated by osteoclasts (Birkedal-Hansen, 1993b).

Interestingly, Each of major cell types of human periodontal tissues is capable of expressing a unique complement of MMP when properly stimulated by bacterial

products (Birkedal-Hansen, 1993a; DeCarlo et al., 1997). Therefore, our study focuses specifically on MMPs pathway.

1.7 Matrix metalloproteinases (MMPs)

The MMPs comprise a large family of at least 25 members in vertebrate and 22 members in human. These proteins can degrade, or proteolyses, all known components of extracellular matrix. They demonstrate some selectivity such that an individual MMP has the ability to degrade a particular subset of matrix protein. MMP family members are products of different gene. However, the protein products are classified by shared functional and structural characteristics. The catalytic core-domain contain the active, protein-degrading ability of proteinase; this domain includes a metal-binding site for Zn^{2+}, the binding of which is essential for proteolytic activity (and explains the word "metallo" in its name). All MMP have pro-domain that serves to keep the enzyme inactive until proteinase activities are required. The pro-domain act as an internal inhibitor of MMP activity, and activation occurs with cleavage of the pro-domain leading to a conformational change to the active form. This activation model was confirmed by the resolution of crystal structure of the catalytic domain of MMP-1 (Lukashev and Werb, 1998). Importantly, the catalytic domain has been thoroughly examined at both the molecular and structural levels, and the information gained has been used in the design of drugs that could inhibit MMP activity. These inhibitors are considered to managing connective tissue destruction.

1.7.1 Classification of MMPs

MMPs are classified on the basis of additional protein domains that contribute to their individual characteristic. The active form of MMP-7 contains only the catalytic domain characteristic of all MMPs. All other MMPs have an additional C-terminal domain with homology to a serum protein called hemopexin. The hemopexin domain is thought to mediate additional protein-protein interactions with substrates and with naturally occurring inhibitors. The roles of additional functional domains include (1) modulating protein interactions important in substrate recognition; (2) providing transmembrane localization (3) providing an alternative cleavage site for MMP activation. In addition, although most MMPs identified to date are secreting enzymes, cell-surface-bound MMPs containing a transmembrane domain (Sato et al., 1996; McCawley and Matrisian, 2000). Domain structure of MMPs and theirs own substrates (Sternlicht and Werb, 2001) as shown in **Figure 1.1** and **Table 1.1,** respectively.

1.7.2 Regulation of MMP functions

MMPs are highly regulated, with both expression, secretion and particularly in activity levels under tight control (Thompson et al., 1994). In general MMPs are expressed at very low levels, however, expression is rapidly induced at times of active tissue remodeling. MMP proteins are transcribed and secreted by the constitutive secretory pathway, except in case of immune cells such as macrophage and neutrophils where MMPs can be stored in, and released from, secretory granules. The activity of MMPs is controlled extracellularly: they are secreted in an inactive, latent

form that requires removal of the pro-domain to attain the catalytically active state. MMP activity is further modulated through interactions with their natural inhibitors, the tissue specific inhibitors of metalloproteinases (TIMPs). There are 4 TIMPs family members described to date, most of which are capable of binding and inhibit the activity of all members of the MMPs family. However, They demonstrate differences in tissue distribution, and different TIMPs differ in their ability to form complexes with the inactive form of MMP and, thus the ability to control MMP activation (Gomez et al., 1997).

1.7.3 MMPs in human periodontal disease

It is widely believed that destruction of the connective tissue attachment of teeth and adjacent alveolar bone observed in patients with chronic adult periodontitis is consequence of host inflammatory reactions initiated in response to bacterial colonization of the subgingival environment (Listgarten, 1987). The consequences of these events are altered levels of specific immunomodulators and the release of inflammatory protease (Birkedal-Hansen, 1993a; Mitchell et al., 1993). Many proteases are capable of degrading a wide variety of substrates including collagen, a major constituent of the extracellular matrix (ECM) within the periodontium. The enhanced activity of these enzymes is thought to be involved in the extensive tissue destruction observed in many chronic inflammatory diseases.

A number of studies have implicated matrix metalloproteinases (MMPs) and other proteases as playing a central role in pathogenesis of periodontal disease. Since, the MMPs are capable of degrading the majority of proteins found within the ECM.

Many reports have demonstrated the presence of specific MMPs including gelatinases A and B (MMP-2 and MMP-9) interstitial collagenases (MMP-1 and MMP-8), and stromelysin (MMP-3) in gingival biopsy specimens and gingival crevicular fluid (Birkedal-Hansen, 1993a; Tervahartiala et al., 2000; Kiili et al., 2002). Previously, the increasing level of MMP-2 and MMP-9 was observed in periodontal patients (Makela et al., 1994) whereas latent MMP-9 was the major gelatinase in GCF and saliva. Recent studies utilizing a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) have shown preferential expression of MMP-3, follow by MMP-1 and MMP-8 in periodontitis-affected tissue (Kubota et al., 1996). The current study also demonstrated an association between increased GCF MMP-8 activity and progressive loss of connective tissue attachment (Lee et al., 1995). In addition, increases in the levels of neutrophil-derived serine proteases, such as cathepsin G and thiol proteases, have also been associated with periodontal tissue destruction (Kunimatsu et al., 1993; Lah and Kos, 1998). Taken together, MMP-2 was found in the gingival tissue of the patients. In addition, increase of active MMP-2 was recently shown in chronic adult periodontitis (Makela et al., 1994; Korostoff et al., 2000). These results strongly suggest the involvement of MMP-2 in periodontal destruction.

1.8 Matrix metalloproteinase-2 (MMP-2)

MMP-2, or Gelatinase A, could degrade extracellular matrix proteins including glycoproteins, proteoglycans, and type IV collagen: the major structure component in basement membrane, type I collagen and also denature collagen as well. Thus MMP-2 is thought to play a crucial role in periodontal destruction and invasiveness and metastasis of various kinds of carcinomas. Most cells of

mesenchymal origin have the ability to synthesize and secrete MMP-2 in latent form (pro-enzyme) but must be further stimulated to enable activation of MMP-2 for proteolytic function (Pulyaeva et al., 1997; Ruangpanit et al., 2001).

Regulation and Activation

Unlike other MMPs, MMP-2 was constitutive expressed by many cell types (Brown et al., 1996). MMP-2 function is controlled at three levels; transcriptional level, activation and inhibition. MMP-2 activation is different from other MMPs, since MMP-2 activation requires the formation of trimolecular complex comprising of MT1-MMP, tissue inhibitor of metalloproteinase-2 (TIMP-2) and MMP-2 on the cell surface (Strongin et al., 1995). Early studies showed that the plasma membrane fraction from cultured cells could activate pro-MMP-2 and was inhibited by chelating agents (Azzam and Thompson, 1992). Therefore, the pro-MMP-2 activator was thought to be a member of the MMP family and more specifically a membrane-associated protein, which is now well documented as the subfamily of cell surface-associate membrane type MMPs (MT1-MMP).

According to MMP-2 plays role in many diseases. For instance, the increased level of MMP-2 expression has been associated with the pathogenesis of joint disorder and cancer (Smolian et al., 2001; Srinivas et al., 2001). In addition, an increasing ratio of active to latent MMP-2 corresponds with the metastasis potential and aggressiveness of many tumor cells (Thompson et al., 1994; Gilles et al., 1997).

1.9 Problem and hypothesis

Many local factors are produced by host cells in response to microorganism invasion during periodontal disease. The biological molecules that responsible for extracellular matrix degradation are host enzymes. These enzymes can be found in both gingival crevicular fluid and especially in gingival tissue itself. The most important host enzymes are MMPs. Although a number of reports are available on the presence of elevated levels of specific MMPs in latent and active forms, such as MMP-1 and MMP-8, MMP-2 and MMP-9, and MMP-3. The amount and the actived MMPs indicated disease severity. From previous reports bacterial products from oral anaerobes; *Porphyromonas gingivalis* can induce MMP-1, MMP-3 MMP-9 activation (DeCarlo et al., 1997). Recently, MMP-2 in periodontitis lesion was elevated and also the active form of MMP-2 was significant higher than normal tissues (Makela et al., 1994; Korostoff et al., 2000). These results can lead directly to tissue destruction in periodontitis Interestingly, the existing of a prevail periodontal pathogen especially gram-negative anaerobes involved in periodontal disease exert a part of their destructive effect by triggering and inducing host cell to elevate their secretion of MMPs (Ding et al., 1995; DeCarlo et al., 1997). They possibly play an important role in MMP function by direct or indirect enzymes activation. This study focuses on MMP-2 activation which possibly processed by bacterial products from mixed periodontal pathogen and also the only predominantly species, *P. gingivalis.* Together with the effect of LPS on MMP-2 activation was included.

Although, MMP-2 activity was highly regulated, with both expressions, secretion and activation under tight control as like as other MMPs. Recently, the novel processing MMP-2 by serine protease activity from bacterial lipopolysaccharide; *E. coli* endotoxin (Takeda et al., 2000). These results indicated that MMP-2 activation not only processed by MT1-MMP dependent pathway but also the serine protease activity from bacterial lipopolysaccharide. The MMP-2 activation within the relation to gram-negative anaerobes cultivated from periodontal pockets is less well defined. The purpose of this study was to determine the effects of bacterial products of gram-negative anaerobes cultivated from periodontal pockets on MMP-2 activation in human periodontal ligament cells and gingival fibroblasts.

We hypothesized that bacterial products secreted by periodontopathogen which most of them are gram-negative anaerobic bacteria and predominantly colonized in periodontal pocket may involve in MMP-2 secretion and MMP-2 activation in periodontal disease. The increasing of MMP-2 level and also MMP-2 activation lead to the progression of adult periodontitis. However bacteria products and LPS may involve MMP-2 activation by direct and/or indirect activation.

1.10 Specific Aims

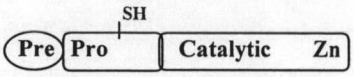
1. To study the relationship between supernatant of anaerobic bacteria and MMP-2 activation
2. To study the relationship between supernatant of *P. gingivalis* and MMP-2 activation
3. To examine the mechanism of MMP-2 activation by *P. gingivalis* LPS

The study was performed with two kinds of primary cell culture; human fibroblasts derived from gingival tissue and periodontal ligament tissue. To proof these hypotheses three distinct approaches were organized. The first approach, HGF and HPDL were stimulated with bacterial products, mixed anaerobes, cultivated from periodontal pockets. The second approach, cells were stimulated with bacterial products secreted from *P. gingivalis.* The last approach, cells or condition medium of cultured cells were stimulated with bacterial endotoxin extracted from *P. gingivalis.* Zymography was used to analyze MMP-2 activation. RT-PCR and western blot were used to analyze specific gene expression (MMP-2) at transcriptional and translational level, respectively. In bacterial culture, at first bacteria were grown as mixed anaerobes to collect supernatant of mixed anaerobes. Then, *P. gingivalis* were isolated from periodontal pockets and bacteria were identified by two methods; PCR and Rapid ID 32A.

Figure 1.1 Domain structure of MMPs. Pre, Signal sequence; **Pro,** Propeptide with a free Zinc-ligating thiol (SH) group, **F,** furin-susceptible site; **Zn,** zinc-binding site; **II,** collagen-binding fibronectin type II insert; **H,** hinge region; **TM,** transmembrane domain, **C,** cytoplasmic tail; **GPI,** glycophosphatidyl inositol-anchoring domain; **C/P,** Cysteine/ proline; **IL-1R,** interleukin-1 receptor. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond

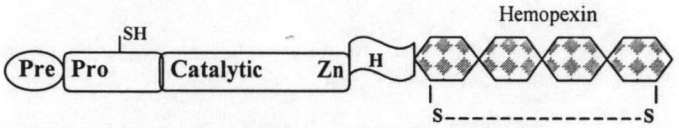
Figure 1.1 Domain structure of MMPs

A) Minimal Domains MMPs (MMP-7, MMP-26)

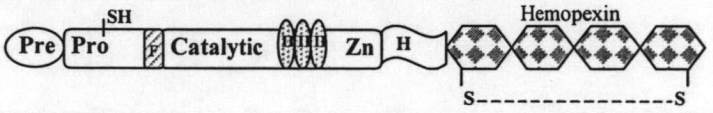


B) Simple Hemopexin Domain-Containing MMPs

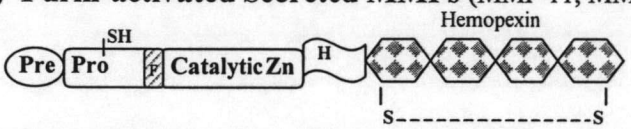
MMP-1, MMP-8, MMP-13, MMP-18, MMP-3, MMP-10, MMP-27
MMP-12, MMP-19, MMP-20, MMP-22



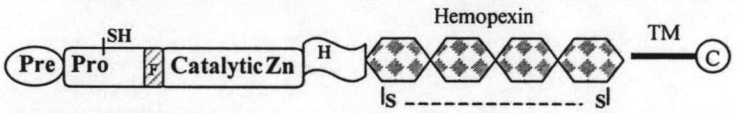
C) Gelatin-binding MMPs (MMP-2, MMP-9)



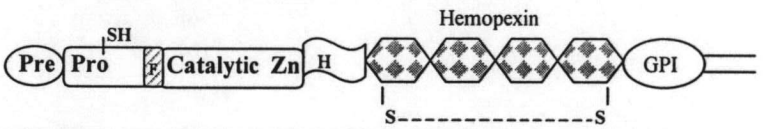
D) Furin-activated Secreted MMPs (MMP-11, MMP-28)



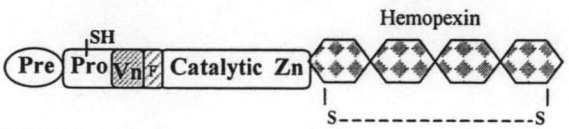
E) Transmembrane MMPs (MMP-14, MMP-15, MMP-16, MMP-24)



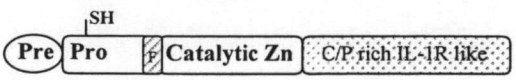
F) GPI-linked MMPs (MMP-17, MMP-25)



G) Vitronectin-like insert linker-less MMPs (MMP-21)



G) Cysteine/Proline-rich IL-1 Receptor-like Domain MMPs (MMP-23)



A) Minimal Domains MMPs

MMP-7/matrilysin, MMP-26/endometase

B) Simple Hemopexin Domain-Containing MMPs

MMP-1/collagenase-1, MMP-8/ collagenase-2,
MMP-13/collagenase-3, MMP-18/collagenase-4,
MMP-3/stromelysin-1, MMP-10/stromelysin-2,
MMP-27, MMP-12/metalloelastase,
MMP-19/RASI-1, MMP-20/enamelysin-2,
MMP-22/CMMP

C) Gelatin-binding MMPs

MMP-2/gelatinase-A, MMP-9/gelatinase-B

D) Furin-activated Secreted MMPs

MMP-11/strmelysin-3, MMP-28/epilysin

E) Transmembrane MMPs

MMP-14/MT1-MMP, MMP-15/MT2-MMP,
MMP16/MT3-MMP,MMP24/MT5-MMP

F) GPI-linked MMPs

MMP-17/MT4-MMP, MMP-25/MT6-MMP

G) Vitronectin-like insert linker-less MMPs

MMP-21/XMMP

G) Cysteine/Proline-rich IL-1 Receptor-like Domain MMPs

MMP-23

Table 1.1 Common matrix metalloproteinase substrates. MMPs, are able to degrade all components of extra cellular matrix (ECM) proteins. This table shows a broad spectrum of substrate specificity of each MMPs. Base on substrate specificity, MMP members are classified by their degradative enzyme functions. These enzymes can regulate cell behavior by cleave a wide variety of non-ECM substrates, including cytokines, growth factors and their receptors.

Table 1.1 Common matrix metalloproteinase substrates

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
ECM Proteins															
Aggrecan	+	+	+	+	+	+	+		+	+	+				
collagen I	+	+	-	+	+	-			+	+	+		+	+	-
collagen II	+				+	-				+	+				-
collagen III	+	+	+	-	+	-	+			+	+	+			
collagen IV	-	+	+	+	-	+	+	-	+	-				+	+
collagen V	-	+	+	-	-	+	+			-					
collagen VI	-	-	-			-				+					
collagen VII	+	+	+												
collagen VIII	+									+					
collagen IX	-	-	+							+					
collagen X	+	+	+	-						-					
collagen XI	+	+	+			+				+					
collagen XIV	-		-	-		+									
Decorin	-	+	+	+		+									
Elastin	-	+	+	+		+	+		+						-
Elastin/Nidogen	+	+	+	+					+		+				
Fibrillin		+	+			+			+	+	+				
Fibronectin	+	+	+	+	-	-	+		+	+	+	+		+	+
Fibulins		+		+											
Gelatin I	+	+	+	+		+	+		+	+	+			+	+
IGFBPs	+	+	+					+							
Laminin	+	+	+	+		+		-	+	+	+				-
Link Protein	+	+	+			+	+								
Myelin Basic	+	+	+			+			+						
Osteonectin		+	+			+				+					
Tenascin	+	+	+			-								+	
Vitronectin	+	+	+			+			+		+				

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
Other Proteins															
α1-AC	+	+	+			-									
α2-M	+	-	+		+	+		+	+	+	+				
α1-PI	+	+	+	+	+	+		+	+		+				+
casein	+	-	+	+		+	+	-		+				+	-
C1q	+	+	+		+	+				+					
E-cadherin			+	+											
Factor XII			+		-				+	+	+				
Fibrin	+	+	+			+					+				
Fibrinogen	+	+	+	+	+	+	+		+	+	+				+
IL-1α	-	-	-			-									
IL-1β	+	+	+			+									
ProMMP-2			+								+	+			
ProTGFβ		+	+			+									
ProTNFα	+	+	+	+		+			+		+				
Plasminogen		+	+	+		+			+						-
Substance P		+	+		+	+									
T kininogen		-	+												

Abbreviation: α1-AC, α1-antichymotrypsin
α2-M, α2-macroglobulin
α1-PI, α1-proteinase inhibitor