CHAPTER 5

GENERAL DISCUSSION AND FUTURE WORKS

Discussion

In the present study, I have undertaken a comprehensive analysis of MMP-2 activation by bacterial products; mixed anaerobe supernatant, *P. gingivalis* supernatant and *P. gingivalis* LPS in cultured cells, HPDL and HGF. This study focuses specifically on MMPs pathway in periodontal destruction by directly demonstrated the critical relationship between bacterial products and MMP-2 activation. Since, periodontal disease is associated with a complex microflora. Thus, the beginning of this study investigated the effect of mixed infection of anaerobes cultivated from periodontal pockets on MMP-2 activation. Treatment of HGF and HPDL cells with culture supernatant of mixed anaerobes resulted in the production of active MMP-2 in fully active form with a molecular mass of 62 kDa, whereas non-treated control cultures did not show the active form.

The second part focuses on *Porphyromonas gingivalis (P. gingivalis).* Because one of the most significant bacteria associated with progressive form of periodontitis is *P. gingivalis.* The bacteria were isolated from periodontal pockets and the culture supernatants of pure *P. gingivalis* were prepared. Treatment of HGF and HPDL cells with culture supernatant of *P. gingivalis* resulted in the production of active MMP-2 in fully active form with a molecular mass of 62 kDa. These results were similar with the results obtained from the supernatant from mixed anaerobes, whereas non-treated

control cultures did not show the active form. These data indicated that *P. gingivalis,* one of the critical periodontopathogen, secreted bacterial products that contain the ability to activate MMP-2. The activation process was inhibited by the presence of chelating agents, 10 μM phenanthroline, which is a Zn^{2+} specific chelator. In contrast serine protease inhibitors (PMSF, aprotinin) and cysteine protease inhibitor (leupeptin) did not interfere the activation process. Importantly, the MMP-2 activation process was completely inhibited by NF-kB inhibitor (APDC). The latter results suggested that MMP-2 activation is NF-kB dependent pathway. These collective results indicate the important of MMP-2 activation that required another Zn^{2+} dependent enzyme, possibly MT-MMP at cell membrane. Since this observation shown the bacterial products at non-toxic level could induce MMP-2 activation and this process could be inhibited by the presence of EDTA. The reason for the inhibition effect of EDTA is possibly results from its metal chelating agent that can competitively bind to the metal ion (Zn^{2+} or Ca^{2+}) in the system. From previous study, MMP-2 activation required the function of another MMP, MT1-MMP (Azzam and Thompson 1992). Thus, the presence of EDTA possibly led to MT-MMP dysfunction, then the latent MMP-2 could not be processed to active form. Our observations demonstrated the indirect pathway of periodontal destruction by microbial products, which can stimulate the dissolution of ECM molecules by activate host derived matrix melloproteinases. These results were similar to previous report that showed the mechanism of collagen dissolution by *P. gingivalis* culture medium through the activation of MMP precursors, particularly MMP-1, MMP-3 and MMP-9 (DeCarlo, Windsor et al. 1997). *P. gingivalis* supernatant could induce MMP-2 activation was similar to mixed anaerobe supernatant. The mechanisms of MMP-2 activation by mixed anaerobe supernatant and *P. gingivalis* supernatant were inhibited by chelating

agents. The results indicated that *P. gingivalis,* which is one of the most significant putative periodontopathogens, could secrete bacterial virulence factors and subsequently activated MMP-2 for degradative function. It is possible that other species colonized in periodontal pockets may posse ability to secrete virulence factors that involved in MMP-2 activation.

Many studies looking for identification the surface-associated component of bacterial cell wall, which have ability to activated host enzymes in periodontal tissue destruction. One of the most significance in periodontal disease activity is not only the level of MMP-expression but also the level of active MMP, especially proMMP-2 and active MMP-2. The MMP activity is markedly increased in gingival tissue and gingival crevicular fluid (GCF) of periodontitis patients (Overall and Sodek 1987), whereas the presence of a matrix metalloproteinase inhibitor could reduce the collagen degradation fragments in GCF (Golub, Lee et al. 1997). Recently reported, preparation of whole cell sonicated, formaldehyde-fixed cells and outer membrane fraction (OMF) of *Treponema lecithinolyticum* posses ability to process latent MMP-2 to fully actived MMP-2 (Choi et al. 2001). Furthermore, pretreatment of PMSF before stimulation and heat treatment of bacterial preparation could not activate MMP-2. These data indicated that LPS, which is the major component of outer membrane of these bacteria did not seem to be involved because heat-denatured cell sonicated failed to activate proMMP-2. From these data suggested that MMP-2 was activated by the components which molecules were heat sensitive molecules and these molecules may be proteins in nature.

These results suggested that low amount of bacterial products acted as triggering molecules and regulated host enzymes to function. As we try to proof the capability of bacterial supernatant-induced direct MMP-2 by incubated conditioned medium of cultured cells with bacterial supernatant. It was found that bacterial supernatant could not induce the MMP-2 activation in condition medium (data not shown). The latter evidence revealed that MMP-2 activation in the presence of bacterial supernatant is NF-kB-dependent pathway, that means MT-MMP may be required. An imbalance of MMPs and tissue inhibitor of metalloproteinases (TIMPs) may disturb the regulation of MMP function. Recent study has shown that protease releasing from *P. gingivalis* could directly degrade TIMP-1 (Grenier and Mayrand, 2001). In addition sonicated extract of *P. gingivalis* could induce MMP-2 production and inhibit TIMP-2 production (Nakata et al., 2000). These data strongly supported that dysregulation of MMP function by an imbalance of MMPs and endogenous TIMPs (MMP-2/TIMP-2) may lead to the accelerated breakdown of periodontal connective tissue.

In the third part, *P. gingivalis* LPS was isolated. Treatment of HGF and HPDL cells with *P. gingivalis* LPS resulted in the production of active MMP-2 in fully active form with a molecular mass of 62 kDa, whereas non-treated control cultures did not show the active form. The activation process was inhibited by the presence of serine protease inhibitors (PMSF, aprotinin). In contrast 10 μM phenanthroline, which is a Zn^{2+} specific chelator, could not block the activation process. It was found that in the presence of APDC (NF-kB inhibitor) MMP-2 activation was partially inhibited. Our results also demonstrated the direct activation of MMP-2 by *P. gingivalis* LPS by mean of incubated the conditioned medium of cultured cells with *P. gingivalis* LPS

for 24 hours. The *P. gingivalis* LPS induced direct MMP-2 activation, this process was inhibited by serine protease inhibitor. These results indicated that *P. gingivalis* LPS induced MMP-2 activation occurred by serine protease activity of *P. gingivalis* LPS. These data suggested that *P. gingivalis* LPS induced MMP-2 activation via both NF-kB dependent and direct activation processes. These observations are similar to the recent study demonstrated that *E. coli* LPS can directly activate purified MMP-2 *in vitro* (Takeda et al. 2000). These data suggested that the LPS, which is one of the releasing bacterial products from *P. gingivalis* colonized around the root surfaces, could activate MMP-2 to be an active enzyme. Microenvironment of periodontal pocket has occupied by a community of complex microorganism and their products. Other species may have involving effects on periodontal tissue degradation in synergistic pathway of tissue degradation. In addition, a numerous cell types in periodontal tissues possess a capability to response in an individual manner that may enhance or attenuate the collective effect of LPS on connective tissue destruction. Recent study has shown the effects of lipopolysaccharide of *Treponema denticola* on host destructive processes by induced osteoclastogenesis and matrix metalloproteinase expression (Choi et al. 2003). However the amount of LPS stimulating dose for peripheral mononuclear cells was lower than fibroblasts, the reason of this difference may be results from the different sensitivity of these cells. LPS stimulates peripheral blood mononuclear cell (PBMC) in amount of nanogram range whereas LPS stimulating fibroblast was occurred in amount of microgram range.

Although human periodontal disease is associated with a complex microflora, relatively few species may be responsible for the transition of gingivitis lesion into a periodontitis lesion with progressive destruction of the periodontal tissues. Except for

P. gingivalis other pathogenic bacteria are known to colonize by variety of highly specific mechanism such as *Veillonella* species followed by bacilli, including *Fusobacterium, Prevotella* species, which accumulate in succession, being followed in climax community by the spirochetes (Theilade 1984a; Theilade 1984b) Later in plaque maturation, the lectin-like interaction mediated coaggregation of *P. gingivalis* and *F. nucleatum* (Kinder and Holt 1989). Thus, coaggregation between inter-species and the mechanism by which one bacteria-species adheres to another or to host tissue cells may promote multiple species of bacteria to colonize in periodontal pockets. In addition, its adherence activity and the interaction of bacterial products of multiple species may synergistically enhance the virulence of these bacteria. Recently, it has been shown that the most significant bacteria associated with periodontitis are *A. actinomycetemcomitans, P. gingivalis,* and *B.* (Zambon, 1996). Thus, the interaction between complexity of these species may influence not only the pathogenicity of these bacteria but also a large number of host derived cytokines which served as inflammatory host responses (Gemmell and Seymour 1994). Previously, it has been found that *A. actinomycetemcomitans*, *P. gingivalis* can elevate inflammatory cytokines in human gingival eithelial cells (Sfakianakis et al. 2001; Yamada et al. 2002). These results indicate that periodontal pathogens and inflammatory cytokines play an important role in tissue destruction and disintegration of extracellular matrix components in periodontal diseases. Thus, activation of MMPs may be one of the distinct host degradative pathways in the pathogenesis of periodontitis. (Chang et al., 2002). Particularly, IL-1β, IL-6, IL-8, IL12, TNF-α which known as potent inflammatory regulator in connective tissue destruction (Ishida et al. 1993; Tatakis 1993; Yoshimura et al. 1997; Lertchirakarn et al. 1998; Graves et al. 2001; Yun et al. 2002). The remaining research questions are still await such as the influence of

bacterial products and or LPS on expression level of host proteins, which associated with MMP-2 activation including of MT1-MMP and TIMP-2. Other host proteins, which responsible for LPS; CD14 and Toll like receptors-2 and, -4 (TLR-2 and -4), should be clarified.

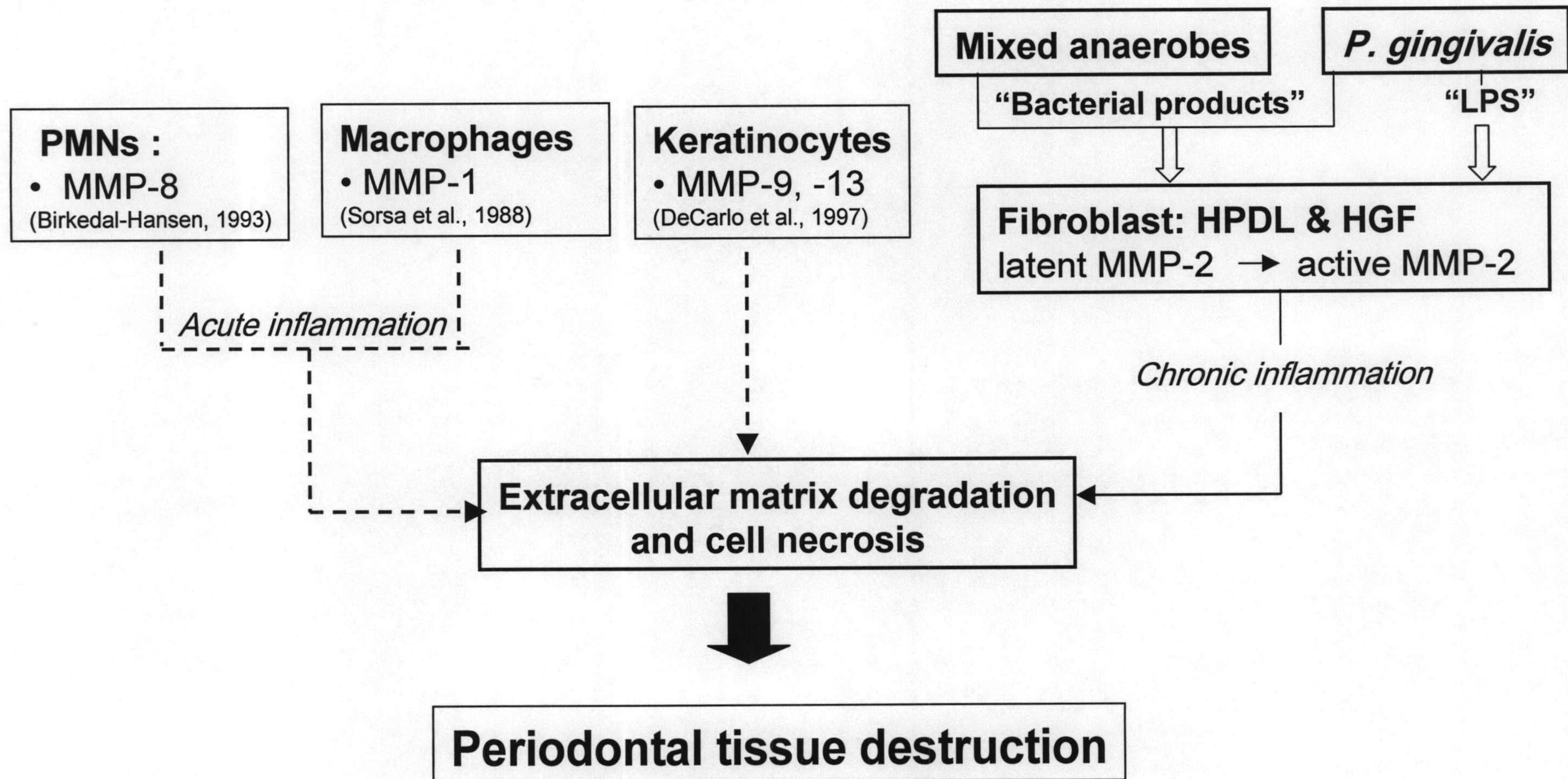
Conclusion

This present study observed MMP-2 activation by bacterial products; mixed anaerobe supernatant, *P. gingivalis* supernatant and *P. gingivalis* LPS in HPDL and HGF. The activation of MMP-2 by bacterial products in vitro demonstrated a possible pathogenic mechanism in periodontal disease.

Figure 5.1 MMPs in periodontal tissue destruction

It has been reported that MMP-1, MMP-8, MMP-9 and MMP-13 associated with periodontal tissue destruction, as shown on the left side, (Sorsa et al., 1988; Birkedal-Hansen, 1993; DeCarlo et al., 1997). MMP-2 was synthesized and secreted by fibroblasts, which are major cells in periodontal tissue. MMP-2 was tight regulated for its degradative function. This study focuses on MMP-2 activation by bacterial products from gram-negative bacteria, especially *P.gingivalis.* The experiments in this study directly demonstrated the involvement of MMP-2 in periodontal disease. It was found that bacterial products from either mixed gram-negative anaerobes or *P. gingivalis* , and lipopolysaccharide (LPS) of *P. gingivalis* could induce MMP-2 activation. Since MMP-2 could degrade collagen type IV but recently it has been shown that it can degrade type I collagen and denatured collagen (Tournier et al., 1994; Aimes and Quigley, 1995).The process of MMP-2 activation is a crucial role for its function. Thus, MMP-2 possibly play an important role in chronic destructive of periodontal disease, as shown on the right side.

Figure 5.1 Model of MMPs in periodontal tissue destruction



FTURE WORK

Base on the results of these studies, bacterial products of mixed anaerobes and pure *P. gingivalis* and also the endotoxin of *P. gingivalis* could activate MMP-2 in HGF and HPDL cells.

Therefore, I hypothesize that

1. There are much more than one component in the supernatant could activate MMP-2.
2. The activated MMP-2 may associate with the up-regulation of MT1-MMP.
3. The activated MMP-2 may associate with the down-regulation or the inactivation of TIMP-2.
4. Bacterial products, such as bacterial mixed anaerobe supernatant or pure culture supernatant from other species may effect on other cell types (keatinocyte, monocyte, etc.) which have a possibility to expose these bacterial products. These data may complete the other mechanisms of periodontal tissue destruction.
5. The influence of host protein expression; MT1-MMP, TIMP-2, CD14 and TLR-2 and-4.

Elucidating factors that influence the development and progression of connective tissue destruction is critical to the development of effective therapies for patients with periodontitis. Importantly, a better understanding of the pathogenesis of periodontitis has resulted in pharmacotherapeutic advancements, addressing both the

microbes and the host response, leading to improved management of this chronic progressive disease by the dental practitioner. The adjunctive use of host-modulatory agents can enhance therapeutic responses, slow the progression of the disease, and allow for more-predictable management of patients.