

CHAPTER 4

DISCUSSION AND CONCLUSION

Periodontal ligament (PDL) fibroblasts play an important role, not only in the formation and maintenance of periodontal ligament, but also in the process of repair, remodeling, and regeneration of the adjacent alveolar bone and cementum (Wise et al., 1992; Hou and Yaeger, 1993; Lackler et al., 2000). Importantly, fibroblasts are a prominent cell type residing in the PDL. They are candidate host cells, which are activated by bacterial antigens, after microbes or their products have gained egress into the periradicular tissues. *E. faecalis* is the most commonly found microbe in endodontically failed teeth, having been detected, using PCR analysis, in 67-78% of root-filled teeth associated with periradicular lesions (Rocas et al., 2004; Siqueira and Rocas, 2004; Gomes et al., 2008). Several studies have reported that bacteria or their products are also involved in MMP-2 activation (Choi et al., 2001). An increased level of MMP-2 was observed in gingival crevicular fluid from teeth with periradicular lesions (Belmar et al., 2008).

This study demonstrates that *E. faecalis* supernatant and heat-killed *E. faecalis* of ATCC 19433 and ATCC 14506 can elevate MMP-2 expression and activation in cultured fibroblasts isolated from human periodontal ligament. The response of fibroblasts in terms of MMP-2 activation depends on the concentration of stimulants. This evidence implies that fibroblasts in PDL can directly use the active MMP-2 in the process of extracellular matrix degradation, leading to periradicular tissue destruction in apical periodontitis.

Two reference strains of *E. faecalis*, ATCC 19433 and ATCC 14506, were used as stimulants to determine whether the differences in the strain types of *E. faecalis* affect MMP-2 activation. This study found that the ability of *E. faecalis* ATCC 19433 to induce MMP-2 expression and activation was similar to that of *E. faecalis* ATCC 14506.

E. faecalis is known to produce gelatinase (Bleiweis and Zimmerman, 1964). Previous studies reported that the molecular weight of gelatinase from *E. faecalis* was approximately 33 kDa (Makinen et al., 1989), or 35 kDa (Su et al., 1991). In order to assure that the MMP-2 identified in this study was the result of the activation of fibroblasts by *E. faecalis* supernatant, equal amounts of *E. faecalis* supernatant and culture media containing stimulated fibroblasts were used to compare gelatinolytic activity by gelatin zymography. A clear band which would indicate gelatinolytic activity from supernatant could not be detected (see Appendix) whereas the clear bands of MMP-2 (72, 68, and 62 kDa) could be detected in the culture media. This result might be caused by the amount of gelatinase in the supernatant being too low for its activity to be detected. However, the amount of supernatant was sufficient to stimulate cultured fibroblasts to generate and secrete MMP-2, and activated MMP-2. Therefore, in future studies, an amount of *E. faecalis* supernatant greater than 15 μ l should be used to determine gelatinase activity by gelatin zymography.

The results of *E. faecalis* supernatant on MMP-2 expression and activation were in agreement with those of other studies (Pattamapun et al., 2003; Tiranathanagul et al., 2004), which revealed that bacterial supernatant of some bacteria such as *P. gingivalis* and *A. actinomycetemcomitans*, possessed an ability to activate MMP-2. Heat-killed *E. faecalis* induced MMP-2 activation, similar to the

finding of a previous study (Nakata et al., 2000), that pro-MMP-2 and active-MMP-2 were increased after pulpal fibroblasts were treated with sonicated bacterial extracts of *Prevotella endodontalis* and *P. gingivalis*. Furthermore, a study by Choi et al. (2001) showed that whole cell extracts of *Treponema lecithinolyticum* could induce MMP-2 activation in gingival fibroblasts and periodontal fibroblasts.

The mRNA expression of MMP-2 was increased after stimulation with either *E. faecalis* supernatant or heat-killed *E. faecalis*. This result suggests that *E. faecalis* supernatant and heat-killed *E. faecalis* affect the transcriptional level of MMP-2. These effects on the transcriptional level of MMP-2 corresponded to the findings of a previous study (Yanagisawa et al., 2005) that *Helicobacter pylori* could up-regulate MMP-2 expression in human enterohepatic cells.

This study performed the test of gene expression after incubation for 48 hours according to the previous studies revealed that, mRNA expression of MT1-MMP (Pattamapun et al., 2003) and TIMP-2 (Tiranathanagul et al., 2004) could have been altered significantly different after stimulation HPDL cells with bacterial supernatant for 48 hours. However, the induction of gene expression might occur before this time-point. Therefore, the kinetic studies should be investigated and Real-time PCR should be performed to detect gene alteration in the future study.

The results of the present study show increased MMP-2 activation both from *E. faecalis* supernatant and heat-killed *E. faecalis* stimulation. However, distinct MMP-2 activation mechanisms resulting from *E. faecalis* supernatant and heat-killed *E. faecalis* have been observed. *E. faecalis* supernatant appears to act on TIMP-2 molecules by increasing the TIMP-2 protein level in the cultured media while the

effect of heat-killed *E. faecalis* on TIMP-2 molecules appears to decrease the TIMP-2 protein level.

TIMP-2 plays a dual role in the regulation of MMP-2 activation, functioning both as an activator and an inhibitor of MMP-2 in a concentration-dependent manner (Goldberg et al., 1989; Hernandez-Barrantes et al., 2000; Wang et al., 2000). At low to intermediate concentrations, a TIMP-2-free MT1-MMP can effectively activate pro-MMP-2 (Lu et al., 2004), whereas at high concentrations, all of the cell-surface MT1-MMP undergoes complex formation with TIMP-2, thereby inhibiting pro-MMP-2 activation (Strongin et al., 1995; Kinoshita et al., 1998).

The increase in the expression of TIMP-2 when fibroblasts are stimulated with supernatant and the decrease in the expression of TIMP-2 when the fibroblasts are stimulated with heat-killed *E. faecalis* may be caused by distinct and different molecules in the supernatant and heat-killed *E. faecalis*, respectively. Supernatant consists of bacterial secretory products, such as proteinase and extracellular products, while heat-killed *E. faecalis* consists of bacterial cell wall fractions, such as peptidoglycan and lipoteichoic acid (LTA). LTA can induce inflammatory mediators, such as TNF- α , NO (Baik et al., 2008). These factors may participate in MMP-2 activation and may cause the difference in stimulating mechanism.

Thus, further studies are planned to investigate and identify the types of molecules, purify them and use them to stimulate fibroblasts directly. The exact mechanism of MMP-2 activation will be investigated using specific inhibitors to block molecules involved in possible pathways on MMP-2 activation. To confirm that the increase in the expression of TIMP-2, when fibroblasts are stimulated with *E. faecalis* supernatant, leads to MMP-2 activation in cultured fibroblasts, further studies

will be performed by titration of the endogenous TIMP-2 with a specific antagonist of TIMP-2. After that, a purified exogenous TIMP-2, of the same concentration as the titrated endogenous TIMP-2, instead of *E. faecalis* supernatant, will be used to stimulate fibroblasts, and MMP-2 activation will be analyzed using gelatin zymography. MMP-2 activation by *E. faecalis* supernatant and exogenous TIMP-2 will be compared. For the investigation of the effect of heat-killed *E. faecalis* on MMP-2 activation in cultured fibroblasts by reduction of TIMP-2, a specific antagonist of TIMP-2 will be used to replace heat-killed *E. faecalis*. In addition, confirmation of the effect of *E. faecalis* on MMP-2 expression at the transcriptional and translational levels, respectively, actinomycin D (an inhibitor of RNA synthesis) and cycloheximide, (a protein translation inhibitor), will be used.

MMP-2 and TIMP-2 protein expression in the culture medium indicated that molecules were secreted by the cells. However, MMP-2 activation has also been found at the cell surface (Strongin et al., 1995). Therefore, further investigations should attempt to detect ratios among MMP-2, MT1-MMP and TIMP-2 molecules in both the lysate cell and the culture medium, as described in other studies (Jo et al., 2000; Tiranathanagul et al., 2004). To determine the concentration of each molecule, titration should be performed with known concentrations of specific antagonists.

Conclusion

In summary, *E. faecalis* could induce MMP-2 expression and activation in cultured fibroblasts from human periodontal ligament. The relationship between *E. faecalis* and MMP-2 may play an important role in periradicular tissue destruction in endodontically failed teeth with periradicular lesions, infected by *E. faecalis*.