CHAPTER V

DISCUSSION AND CONCLUSION

It is known that host immune response employs TLR and non-TLR pathways to recognize pathogens and commensal bacteria. This recognition leads to expression of mediators which limit microbial invasion. In the oral cavity, the epithelial cell is the first layer exposing to many bacterial pathogen-associated molecular patterns (PAMPs), so its innate immune response must be highly orchestrated and delicately balanced, in order to maintain homeostasis. In this study, we investigated the expression of TLRs and their role in signaling by HGECs. The results showed that HGECs from healthy gingival tissues clearly expressed mRNA of TLRs 1, 2, 3, 5, 9, 10, and minimally expressed TLR4, but did not express TLRs 7 and 8. However, the expression of TLR4 mRNA was very minimal. This is similar to observations in previous studies using primary HGECs and epithelial cell lines (Kusumoto et al., 2004, Sugawara et al., 2006).

HBD-2 is well recognized as a evidence anti-microbial peptide in the oral cavity and gingival epithelium is a major source of this mediator. In our study, we stimulated HGECs with a variety of highly purified specific ligands and measured their HBD-2 expression by RT-PCR. In line with the TLR mRNA expression, HGECs expressed HBD-2 in response to *P. gingivalis* LPS, poly (I:C), *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, and 5. However, CpG ODN 2006, a potent ligand for TLR9, did not induce HBD-2 expression. Similar non-responding profile to CpG ODN 2006 was also

reported in human gingival fibroblast culture from our laboratory (Mahanonda et al., 2007). The underlying mechanisms of these phenomena need further investigation.

Different culture conditions for HGEC stimulation affected HBD-2 expression. Our result of *P. gingivalis*-induced HBD-2 in HGECs agrees with the study of Taguchi et al. (2006), but in conflict with Krisanaprakornkit et al. (2000). This may be due to the purity of bacterial preparation. The latter study used crude cell wall extracts of *P. gingivalis* whereas our study used highly purified *P. gingivalis* LPS. Furthermore Sugawara et al. (2006) reported TLR4 ligand-stimulated HBD-2 in human oral epithelial cell line, HSC-2. This cell lines was derived from oral carcinoma whereas primary gingival epithelial cells were used in our study. In addition their culture medium was supplemented with 10% FCS but ours was not.

The findings that TLRs on HGECs function as pattern-recognition receptors and signaling molecules, supports their role in the innate immune response against pathogens including bacteria and viruses. Oral plaque bacteria are known to have PAMPs that are recognized by TLRs 2 and 5. For example, *P. gingivalis* LPS and *P. gingivalis* fimbriae are recognized by TLR2 (Asai et al. 2001; Hirschfeld et al. 2001; Zhou et al. 2005) Flagellin of *Treponema denticola* is most likely recognized by TLR5. The expression of TLR3 in HGECs is interesting since TLR3 recognizes double stranded RNA, a by-product of viral replication and transcription (Alexopoulou et al. 2001). Possible role of herpesviruses in etiology and severity of periodontal diseases has been reported (Amit et al., 1992; Contreras et al., 1999; Slots et al., 2005). The presence of TLR3 thus suggests a role of HGECs in antiviral response.

The results of combined stimulation with *P. gingivalis* LPS and TNF-α demonstrated the up-regulation of epithelial HBD-2 expression. Our culture conditions in vitro were designed to imitate periodontal disease conditions where bacterial products and pro-inflammatory cytokines are presence. The increased levels of HBD-2 in our study was in conflict with previous in vivo data which demonstrated the decreased levels of mRNA expression for HBD-2 in periodontitis tissues (Dunsche et al., 2002; Bissell et al., 2004). The possible explanation may be that HBD-2 produced in diseased tissue could be degraded and inactivated by the cysteine proteases cathepsins B (Taggart et al. 2003). In periodontitis, the enhanced expression of cathepsins B could be detected, hence being likely to interfere with HBD-2 production in vivo (Eley et al. 1991). On the other hand, healthy periodontal tissues express HBD-2 and it is thought to be resulted from low challenge of bacterial plaque or the commensals as well as the presence of low levels of pro-inflammatory cytokines in healthy subclinical lesion. Therefore, the interaction between dental plaque commensals with gingival epithelium may provide a beneficial antimicrobial activity and maintain homeostasis in clinically healthy gingival. Once this homeostasis is disrupted, overgrowth of periodontopathic bacteria may emerge and threaten host defense against pathogens, thus leading to periodontal tissue destruction.

Cigarette smoking has a strong association with severity of periodontitis. Nicotine, a major component of cigarette smoke, has several biologic effects to suppress immunological defense mechanism (King et al., 1988; Martin, 1977; Ferson et al., 1979). Our study is the first to report inhibitory effect of nicotine on the innate immune defense of HGECs in terms of anti-microbial function. In the absence of nicotine, the anti-microbial HBD-2 expression in HGECs were markedly up-regulated in response to *P. gingivalis*

LPS and TNF-α. These are key bacterial pathogen products and pro-inflammatory cytokine consistently present in periodontal inflamed tissues and well known for their role in immunopathogenesis of periodontitis. In contrast, when nicotine was added to HGEC culture which were stimulated with *P. gingivalis* LPS and TNF-α, a significant down-regulation of HBD-2 expression was observed. It is obvious that defensive innate mechanisms of HGECs were deteriorated by nicotine and this may lead to disease susceptibility and severity in smokers. Furthermore, it should be noted that suppressive effect of nicotine on HGEC function in the present study are not due to its toxicity. Since the concentrations of nicotine used in our experiments are in the same non-toxic range (0.1-1mM) as those in previous studies (Johnson et al.,1997; Giannopoulou et al.,2001; Wendell et al.,2001). Their results of nicotine (non-toxic range) treated HGECs and human gingival fibroblasts led to IL-1 and IL-8 production (Johnson et al.,1997; Wendell et al.,2001). In addition, the data from our laboratory demonstrated nicotine-induced IL-8 production from HGECs when stimulated with *P. gingivalis* LPS and TNF-α combination. The inhibitory effect of nicotine on HGEC function may be due to the decreased TLR2 expression on HGECs upon nicotine exposure (unpublished data) and the direct pathobiological effects on structure and function which is possibly via neuronal nicotinic acetylcholine receptors expressed on HGECs (Arredondo et al. 2005). However, the underlying mechanisms of how nicotine suppressed HBD-2 expression or enhancement of IL-8 production in stimulated HGEC culture, required further study.

In conclusion, this study demonstrates that HGECs express mRNA of TLRs 1, 2, 3, 4, 5, 6, 9, and 10. Triggering with *P. gingivalis* LPS, poly I:C and *S. typhimurium* flagellin, ligands specific for TLRs 2, 3 and 5 respectively, led

to the expression of HBD-2. In contrast, the potent TLR9 ligand CpG ODN 2006 did not induce HBD-2 expression. A wide range of actively functioning TLRs as well as their strategic location suggest a crucial role of HGECs in the innate immune response and maintaining homeostasis of the periodontal tissue. This study shows for the first time the suppressive effect of nicotine on defensive anti-microbial activity of HGECs. In the presence of nicotine, significant suppression of HBD-2 expression in HGECs in response to periodontopathic bacterial agent: *P. gingivalis* LPS and pro-inflammatory cytokine: TNF-α was observed. This present study therefore confirms the harmful effect of cigarette smoking and well supports the concept of smoking being an important risk factor in periodontitis.