CHAPTER 4

DISCUSSION

Results from clinical, *in vivo* and *in vitro* studies indicate that EMD has the ability to induce the regeneration of periodontal tissues, i.e. cementum, PDL, and bone. The potential for EMD to stimulate mineralized tissue formation may in part be influenced by its ability to modulate important regulatory proteins related to both osteoblastogenesis and osteoclastogenesis at the mRNA level or at the protein level. From the study of the mechanism by DNA microarray of EMD in osteoblast-like cell MG-63 demonstrated that EMD is able to modulate a broad range of osteoblast biologic processes: (i) signaling transduction, (ii) transcription, (iii) translation, (iv) cell cycle regulation, proliferation and apoptosis, (v) immune system, (vi) vesicular transport and lysosome activity, and (vii) cytoskeleton, cell adhesion and extracellular matrix production.[153] The molecular mechanisms of regeneration ability of EMD to bone tissues on mRNA level in human osteoblast and osteoclastogenesis are poorly understood. The aim of this *in vitro* study was to determine whether EMD could be effective in regulating the production of OPG, RANKL mRNA expression in osteoblast-like cells MG-63 and osteoclast formation. The cellular model used in the present study was based on immature osteoblasts and co-cultures system. MG-63 cells were chosen because they are easy to culture and they provide extensive cellular material for large scale molecular or biochemical studies on factors capable of regulating the metabolism of osteoblast-like cells. Cultured human osteoblasts are more representative of the human osteoblast phenotype; however, they are more variable and dependent upon the clinical status i.e. age, hormonal status, the presence of osteoporosis of the patient sample. A high correlation in expression profile between the primary human osteoblast cell cultures and the osteosarcoma cell lines indicates that human osteosarcoma cells have a useful role as model systems for the molecular analysis of the regulation of genes associated with osteoblast function.

The MG-63 cell line is one of the most popular cell lines for testing biocompatible materials. This osteosarcoma cell line is derived from a 14-year-old Caucasian male.[78] Cells are hypo-triploid with a modal chromosome number of 66 and exhibit fibroblast morphology. MG-63 have been well characterized, and widely used in biocompatibility tests.[79-82] They show numerous osteoblastic features, including the expression of BMP [83] and osteocalcin.[84, 85] They exhibit

characteristics of bone forming cells including high levels of 1, 25-$(OH)_2D_3$-responsive alkaline phosphatase activity and osteoblast-like regulated synthesis of osteocalcin and collagen type I.[86]

EMD is mainly composed of amelogenin (90%) and non-amelogenin proteins. In addition, EMD contains several as yet unidentified bioactive molecules. EMD induces a process that mimics normal odontogenesis. It is believed that enamel matrix macromolecules participate in the reciprocal ectodermal-mesenchymal signaling and the cell-matrix interactions.[18] From the study of Suzuki *et al.* was found that EMD contains growth factor such as BMP and TGF- β like growth factors which contribute to the induction of biomineralization during periodontal regeneration.[26] EMD is not new for periodontal therapy. Several studies have been attempted to clarify the mechanism of EMD in periodontal tissue including transcriptional, translational, and cellular levels. But the mechanism of action of this substance is still unclear.

The importance of OPG and RANKL as regulators of osteoclastogenesis is well understood. RANKL, a type II transmembrane protein, belongs to TNF ligand superfamily. It has two active forms; membrane-bound and soluble forms. RANKL is necessary for osteoclast development, activation of mature osteoclasts and a decrease in osteoclast apoptosis. RANKL over expression has been shown in patients with malignant osteolysis due to myeloma[142] or bone metastases[143], in the rheumatoid synovial pannus[144], and in rats with osteoporosis due to ovariectomy.[121] RANKL binds to its receptor RANK through the extracellular domain consisting of four receptor-binding loops.[40, 86] Although the soluble and membrane-bound forms both are active, the membrane-bound form has been indicated to be more efficient in inducing osteoclastogenesis *in vitro.*[145] OPG acts as a soluble decoy receptor for RANKL. The biological effects of OPG include inhibition of proliferation, differentiation, survival, and fusion of osteoclastic precursor cell; inhibition of the activation of osteoclasts; and promotion of the osteoclast apoptosis.[97, 117, 136] The ratio of RANKL to OPG levels has been shown to be important in bone formation or bone resorption.[137] For example, increasing RANKL and suppressing OPG expression initiate osteoclast formation.

Although many hormones and cytokines are able to promote bone resorption by influencing osteoclasts, most evidence indicates that the osteoclastic actions are mediated by cells of the osteoblast lineage.[97] Osteoclast precursors express RANK, recognize RANKL through cell-to-cell interaction with osteoblasts/stromal cells and differentiate in the presence of MCSF which is also produced by osteoblasts.[136] OPG expressed by osteoblasts is a soluble receptor for

RANKL and acts as a decoy receptor in the RANK–RANKL signaling system.[137, 138] Once formed, the bone-resorbing osteoclasts participate with the bone-producing osteoblasts in maintenance, formation and remodeling of the bone.

In this study, we used semiquantitative RT-PCR to determine the effects of EMD on OPG mRNA and RANKL mRNA in osteoblasts-like cells MG-63. Our result showed that treatment with EMD (100 μg/ml) downregulated OPG mRNA expression at 0, 12 and 24 h after incubation when compared to the control group. The stimulatory effect of EMD on OPG mRNA expression could be seen at 6 h and at 24 h of incubation with EMD. Our findings showed an increase in RANKL mRNA expression levels at 0, 6, 12 and 48 h after culture when compared to the control group except in 24 h of which its expression is the same as that of the control group.

In time course study, we showed that RANKL/OPG ratio was higher in EMD treated-cells than control group except the 6 h-treated group of which the ratio is lower. The result suggested that EMD downregulated RANKL/OPG ratio after 6 h of culture. This is possibly due to the high expression of OPG mRNA at the indicated time. The present findings possibly explain the underlying mechanisms of the stimulatory effect of EMD on osteoclast formation. In MG-63 cocultured with PBMCs, there were significantly higher numbers of osteoclast-like cells in the cultures treated with EMD compared to the control group. Overall, the present study suggested that EMD increased the number of osteoclast-like cells in the cocultures of MG-63 and PBMCs possibly through OPG-RANKL-RANK regulatory system. These findings are in agreement with the studies of Otsuka *et al*. in 2005 and Itoh *et al*. in 2006 that EMD containing bioactive molecules induces osteoclast formation through RANK-RANKL interactions.[42, 154] However, the composition of Emdogain is not well understood. Kawase *et al*. in 2005 demonstrated that EMD contained TGF-β-like substance and BMP-like substance. It is possible that effects of EMD on RANKL and osteoclastogenesis are related to TGF-β stimulation. TGF-β is considered as an important regulator of osteoblast and osteoclast activity[155] and is also suggested to couple bone formation to resorption during the remodeling cycle.[156, 157] However, until recently its precise role in resorption is unclear. There are conflicting results. For instance, TGF-β has both positive and negative actions on osteoclast formation in co-cultures of osteoblasts and monocytes.[158, 159] It has been reported that TGF-β shows both stimulatory and inhibitory effects on bone resorption in organ cultures and *in vitro* osteoclast formation.[121] They found that a lower concentration of TGF-β enhanced *in vitro* osteoclast formation in the presence of IL-1α, while it inhibited the osteoclast

formation at a higher concentrations. Very recently, TGF-β is reported to stimulate the production of osteoprotegerin[40, 86] by mouse primary osteoblasts, the stromal-like cell line, ST-2, and the osteoblast-like cell line, MC3T3-E1[95, 145]. Therefore, the decrease in osteoclast formation at a higher concentration of TGF-β may be due to the production of OPG, a decoy receptor of RANKL.[40, 86, 143]

In 2002, Koseki *et al*. demonstrated a possible functional link between the signaling of the RANKL and TGF-β1 families. The function of TGF-β is a double-edged manner; they could also accelerate bone remodeling by contributing to bone destruction by osteoclasts as well as bone formation by osteoblasts.[160]

Our studies contradict with the study of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand modulation by enamel matrix derivative in human alveolar osteoblasts in 2006.[41] They revealed that EMD seemed to play a potentially important role in the wound-healing process, as it increases both cell growth rate, essential for the reintegration of lost tissue, and cell differentiation, a key factor in determining the characteristics of the neo-bone. These actions may be through an increase in OPG production and a reduction in RANKL release. They pointed out that EMD also seemed able to create a favorable osteogenic microenvironment. This study did not observe the expression of OPG and RANKL m RNA in the same manner as our study. They are interested in OPG and RANKL in osteogenic microenvironment and osteoblast activity, not osteoclast differentiation. Our results did not observe the trend of OPG and RANKL like them. The different could be due to the study design and cell type. They used ELISA method to detect the protein level from soluble OPG and soluble RANKL while we used semiquantitative RT-PCR to detect the OPG and RANKL mRNA from osteoblast like cell. In our study, we used osteoblast like cell MG-63, while they used primary cells from 5-year old boy. The disadvantage of using primary cells is the low reliability. Many factors could constitue to this variable which depend upon the clinical status as we mentioned earlier i.e. age, hormonal status, and presence of osteoporosis. Therefore, the effects of EMD on gene expression may depend on cell types and stage of differentiation. The study of EMD on bone related mRNA in human periodontal ligament cells[161] showed that EMD downregulated RANKL mRNA levels and no significant changes in OPG mRNA levels were observed. RANKL/OPG ratio was decreased in PDL cell treated with EMD. Therefore, they suggested that the application of EMD in periodontal surgery contributes to the clinical improvements in attachment levels and, perhaps, periodontal regeneration by the

inhibition of bone resorption. The results from this study are not consistent with our results, which may be due to the different in the study design and cell type used. They detected the mRNA level only at 24 h by western blot analysis and used human periodontal ligament cells for their experiment.

However, there are other previous studies on the effect of EMD on osteoclastogenesis which are in agreement with our findings. Otsuka *et al.* in 2005 studied the effects of EMD on osteoclast formation which were performed in mouse marrow co-culture system.[154] The results indicated that EMD induced the formation of osteoclasts through RANKL expressed by osteoblastic cells. The authors suggested that EMD may regulate both bone formation and bone resorption during periodontal tissue regeneration. Another studies of EMD on mouse monocytic cell line, RAW 264.7 revealed that EMD P2 promotes osteoclast cell formation by RANK–RANKL interactions in RAW 264.7 cells.[42] They suggested that EMD is able to stimulate osteoclasts as well as osteoblasts. EMD provides a local environment suitable for bone regeneration in periodontal tissues through bone remodeling activities, such as bone formation and resorption.