CHAPTER 1

INTRODUCTION

1.1 Overview

Root resorption is a complication of maximum concern after luxation and avulsion injuries. The root surface and particularly the cemental protective layer are damaged as a result of the injury and the subsequent inflammatory response. The healing patterns that result depend on the degree and surface area of damage to the root and on the nature of the inflammation. If the damage area is less than 20% of the root surface, a transient ankylosis may occur, which can later be resorbed due to functional stimuli, provided that the tooth in the healing period is stabilized with a splint which allows a minimum amount of mobility, or is non splinted. In larger injuries, a permanent ankylosis is created. The tooth thus becomes an integral part of the bone remodeling system, the resorbing cells being, primarily, osteoclasts. Subsequently, osteoblasts replace the desorbed areas of the root with bone. In children, replacement resorption leads to loss of ankylosed teeth usually within 1-5 years. Ankylosis in young patients can complicate normal bone growth and development, resulting in problems such as malocclusion or supereruption of opposing teeth. In attempt to overcome this resorption, Emdogain had been introduce since 1997. It has been widely used in periodontal treatment to promote tissue regeneration. Its regenerative capacity has been demonstrated in both in vitro and clinical studies. Although antiresorptive effects of EMD on bone and cementum have been suggested in an animal study, the underlying mechanism is still unknown. Bone resorption is mediated by activated osteoclasts through the RANKL/RANK/OPG pathway. Bone remodeling induced by EMD is possibly mediated through this RANKL/RANK/OPG regulatory system. In this study we will examine the effects of EMD on OPG and RANKL mRNA expression in osteoblast-like cell MG-63. In addition, we will investigate the effect of EMD on osteoclastogenesis. Our findings give insight into the mechanism of this substance on osteoclast formation and gene expression in osteoblast.
1.2 Literature review

1.2.1 Emdogain

During tooth development, inner enamel epithelium and Hertwig’s epithelial root sheath secrete enamel matrix proteins which initiate enamel formation, regulate the growth of hydroxyapatite crystals and induce root formation.\textsuperscript{12-14} The roles of these proteins in root formation include initiating cementogenesis, mediating the attachment of cementum to dentin, and inducing periodontal regeneration.\textsuperscript{12-14} Enamel matrix proteins contain amelogenin and nonamelogenin proteins such as tuftelin, ameloblastin, enamelin, enamel proteases, and sulfated enamel proteins. Amelogenins comprise more than 90% of enamel matrix proteins. They are hydrophobic proteins, rich in proline, glutamine, leucine and histidine amino acid.\textsuperscript{15}

The amelogenin gene was mapped to human sex chromosomes X and Y\textsuperscript{16} that allows the use of the amelogenin gene to determine sex in DNA samples in forensics.\textsuperscript{17} Degradation of enamel matrix proteins is mediated by enamel proteases during tooth formation\textsuperscript{12, 13} Today, ectodermal tooth enamel proteins, in the form of a commercial preparation of porcine fetal enamel matrix derivative were introduced in 1997.\textsuperscript{18} The enamel matrix derivative induces a process that mimics normal odontogenesis, and it is believed that enamel matrix macromolecules participate in the reciprocal ectodermal-mesenchymal signaling and the cell-matrix interactions that control and pattern these processes.\textsuperscript{18} The active product consists of hydrophobic enamel matrix proteins extracted from porcine developing embryonic enamel. This product, registered as EMDOGAIN\textsuperscript{®}, has been marketed by Biora, Inc.\textsuperscript{18} There are 2 different forms of EMD, lyophilized form which needs to be mixed with propylene glycol alginate (PGA) before application and a premixed form named Emdogain – gel. As the major component of EMD, amelogenin has been considered as an inert matrix protein to support cell attachment.\textsuperscript{19, 20} Recently, various amelogenin gene products have been shown to actively participate in cell signaling to stimulate matrix formation and mineralization.\textsuperscript{21, 22} It was proposed that EMD acts as a matrix for cells at the regenerative site to promote cell attachment, spreading, proliferation, and production of growth factors, which in turn enhance tissue repair and regeneration.\textsuperscript{23}

Recently \textit{in vitro} study showed that direct contact between EMD and osteoblasts is not required to induce cell proliferation. They suggested that soluble peptides released from EMD may contribute to the stimulating effects of EMD on cell proliferation.\textsuperscript{24}
EMD is not new for periodontal therapy. Several studies attempted to clarify the mechanism of EMD in periodontal tissue including transcriptional level, translational level and cellular level. But the mechanism of this substance is still unclear.

### 1.2.1.1 Emdogain formulation

A marketed enamel matrix derivative (Emdogain®, Biora AB, Malmö, Sweden) received FDA approval and is now available for the treatment of periodontal defects. It is a purified acidic extract of developing embryonic enamel derived from six months-old porcine. Its purpose is to act as a tissue-healing modulator that would mimic the events that occur during root development and to help stimulate periodontal regeneration. There are 2 formulas of EMD, lyophilized form and gel form. The first marketed EMD product was supplied in a lyophilized form and was dissolved in an aqueous solution of PGA prior to use. Another form, Emdogain®-gel is a premixed ready to use formulation of enamel matrix derivative. Emdogain–gel became available to facilitate application. Conventional lyophilized EMD is no longer commercially available. A study using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis shows that Emdogain-gel has the same protein profile as EMD. Both contain mainly amelogenin and its derivatives. However, enamel proteinases, such as enamelysin and kallikrein, are only detected in EMD but not in Emdogain-gel. The enamel proteinases are inactivated by heat treatment.

EMD is mainly composed of amelogenin (90%). Amelogenin are proteins that rich in proline (~30%), glutamine (~13%), leucine (~10%) and histidine amino acid (~8%). They also contain growth factor such as BMP and TGF-β like structure. The proteins are solubilized in a neutral propylene glycol alginate vehicle for delivery to the surgical site, as the amelogenins are not soluble at physiologic pH. The propylene glycol alginate can leaves the surgical site after the application allowing the amelogenin fraction to precipitate into an insoluble extracellular supramolecular aggregate. This amelogenin matrix may then serve as a substrate for the pluripotential cells derived from the surrounding vital periodontal ligament to attach, differentiate and secrete cementum matrix. This product when applied to the root surface in conjunction with surgical periodontal therapy has been shown to promote periodontal regeneration as demonstrated in both animal and clinical experiments.

Amelogenin proteins can be dissolved at acidic or alkaline pH and low temperature. As expected for hydrophobic interactions, the best solubility is obtained at low temperature (1º).
An aqueous solution of PGA was found to be a suitable vehicle for EMD.\textsuperscript{12} The viscosity of PGA decreases under physiological conditions; so EMD is released to precipitate on the exposed root surfaces in the treated area. PGA solutions fulfill the essential requirements of a vehicle to facilitate the application of EMD during periodontal surgery.\textsuperscript{30} Both formulas contain 30 mg EMD protein/ml Propylene Glycol Alginate gel, with a viscosity of about 2.5 Pas. In a blinded randomized controlled multicenter study showed that both forms of EMD had same properties in periodontal tissue improvement.\textsuperscript{31}

There are several studies to supported the safety of EMD, they indicated that EMD was completely desorbed after 4-week period when applied to the root surface\textsuperscript{32} and from immunohistochemical study of human wound healing suggested that the healing and/or remodeling process after surgical periodontal therapy with EMD may be followed a period up to 6 months.\textsuperscript{33} The clinical safety of EMD about human antibodies indicated that there was no increase in these antibodies among the patients.\textsuperscript{34} After approval for clinical use, more than 100,000 patients have been treated with EMD by mid-1999. Throughout this period, no side effect that could be linked to the use of EMD has been observed.\textsuperscript{18} From others study which performed in 10 patients, only a slight, no significant activation of the immune system occurred during the first year following Emdogain application.\textsuperscript{35}

The enamel matrix proteins are highly conserved among mammalian species\textsuperscript{15, 34, 36}, and exposure to these proteins takes place during tooth development in early childhood. Thus, tolerance should normally be induced and the proteins recognized by the immune system as "self" proteins. Therefore, it is reasonable to assume that they are safe for human.

1.2.1.2 \textit{In vitro} studies of Emdogain

In an attempt to understand the mechanisms by which EMD promotes periodontal tissues regeneration, investigators evaluated the effect of EMD on periodontal ligament (PDL) cells, bone cells and cementoblasts in culture model. For effects of EMD to periodontal ligament cells, Gestrelius \textit{et al.} in 1997 suggested that EMD enhanced proliferation of PDL cells, but not epithelial cells. It increased total protein production by PDL cells and promoted mineralized nodule formation of PDL cells. In contrast, EMD had no significant effect on migration or attachment and spreading of PDL cells.\textsuperscript{19} In another study aimed at examining the influence of EMD on the viability, proliferation, and attachment of human PDL fibroblasts to diseased root
surfaces in vitro, it was shown that the viability of PDL cells was negatively affected by higher doses of EMD over time, while lower doses elicited no change when compared with control cultures.\textsuperscript{21}

In 2003, Cattaneo V \textit{et al.} studied the effects of EMD in promoting tissue regeneration of human periodontal ligament fibroblasts. The results indicated that EMD enhances human PDL fibroblast proliferation. Furthermore, the cells in the presence of EMD show morphological changes that make them more similar to cementoblasts than to fibroblasts, suggesting a process of cellular differentiation that could play an important role in periodontal tissue repair.\textsuperscript{37} From the results from scanning electron microscopy indicated that EMD appeared to increase attachment of periodontal ligament fibroblasts to diseased root surfaces. In addition, amelogenin was shown to have a cell-adhesive activity, which may partially explain the therapeutic effect of EMD in periodontal regeneration.\textsuperscript{38}

EMD also promote periodontal regeneration by regulate osteoblasts and osteoclasts. A study examining the effect of EMD on osteoblasts showed that EMD has the ability to regulate cells in the osteoblastic lineage. The ability to do so depends on the state of maturation within the lineage. EMD induced differentiation of mature well-established osteoblasts; however, it had no effect on undifferentiated mesenchymal cells. These results were in contrast to the effect of BMP-2, which induced the differentiation of undifferentiated cells, 2T9 (osteoblast progenitor cells), in the lineage. This indicates that EMD is an osteoconductive agent, rather than an osteoinductive one.\textsuperscript{39} However, \textit{in vitro} studies suggest that EMD may have the ability to induce osteochondral progenitor cells to differentiate. In a multipotent mesenchymal cell line (C2C12), it was shown that EMD converts the differentiation pathway of the mesenchymal cells into osteoblasts and/or chondroblasts.\textsuperscript{40} Recently the study of effects of EMD on growth and osteogenic marker modulation in human mandibular osteoblasts showed that EMD was able to enhance osteoblast cell growth. EMD also reduced RANKL release and enhancing osteoblastic OPG production. They concluded that EMD seemed able to create a favorable osteogenic microenvironment by control OPG and RANKL expression in osteoblast cells.\textsuperscript{41}

The studies of effects of EMD on osteoclasts were performed in RAW 264.7 cells. The results indicated that EMD induces the formation of osteoclasts through interaction with RANKL, while ERK and p38 MAPK may play a critical role in the enhancement of osteoclast formation in RAW 264.7 cells. Another study was performed in mouse marrow culture model. Their results
indicated that EMD induces the formation of osteoclasts through RANKL expressed by osteoblastic cells, and suggest that EMD may regulate both bone formation and bone resorption during periodontal tissue regeneration.  

1.2.1.3 *In vivo* studies of Emdogain

The ability of EMD to generate periodontal regeneration was first demonstrated in monkeys. Eight weeks after reimplanted maxillary premolar monkey’s teeth with EMD showed that EMD is possible to induce regeneration of all the periodontal tissues, acellular cementum, periodontal ligament and alveolar bone, in a way that mimics the normal development of these tissues. They concluded that the mechanism behind the regeneration process seems to be a matrix-cell interaction between the amelogenin aggregate of the developing enamel and cells in the periodontal ligament.

Study of the ability of EMD to regenerate acellular extrinsic fiber cementum was first demonstrated in monkeys. Four lateral incisors from each animal were gently extracted. Immediately after extraction, an experimental cavity was made in each root. The test cavities were treated with crude porcine enamel matrix, and the teeth were re-implanted. The results showed that there is a thick layer of acellular, hard tissue that was poorly attached to the denuded root dentin in control group.

There are several studies to support wound healing and osteopromotive properties of EMD. From study of Sakallioglu *et al.* in 2004, they tested the effect of Emdogain® on periodontal wound healing by soft and hard tissue profile of dog’s periodontium. Wound healing after 28 day period revealed that the amount of new cementum was significantly higher in the EMD-treated group than the control group. The amount of new bone and the rate of bone maturation (the number of osteon) were found higher in the test group than in the control group. They concluded from the study that the enamel matrix proteins have more capacity for stimulating periodontal regeneration via their positive effects on root surface, i.e. regeneration of an acellular type of cementum regeneration and formation of new alveolar bone by accelerated osteoconductive mechanism with application of enamel matrix proteins.

Results from these *in vivo* studies indicate that EMD has both osteoconductive and cementoconductive properties. In addition, it also has a stimulatory effect on bone growth.
To compare the effect of EMD with other treatment modalities or combined with other treatment in case of improvement of periodontal regeneration. Several animal studies were conducted so that the histological and clinical outcomes following treatment with EMD could be compared with those achieved with GTR. Critical-size fenestration-type defects produced surgically in the buccal bone of 4 teeth in 3 monkeys were treated with EMD, GTR, or coronally repositioned flap (control). After 5 months, the results showed that, in the GTR group, new connective tissue attachment and new bone formation had consistently occurred, whereas, in the defects treated with EMD or with coronally repositioned flaps, new attachment and new bone formed to various extents. It was concluded that GTR treatment seems to be more predictable than EMD in terms of periodontal regeneration. Using a similar research model, the same investigators evaluated the effects of treating intrabony defects with EMD, GTR, or combined EMD and GTR 6 weeks after intrabony defects were surgically produced in 3 monkeys. Coronally repositioned flaps were used as the control. After 5 months, the monkeys were killed, and descriptive histological evaluation of the healing was made. In the control group, the healing was characterized by a long junctional epithelium and limited periodontal regeneration at the bottom of the defect. Recently there was a study compare between GTR and EMD modalities in treatment of Class III furcation in dogs. This study investigated both histologically and histometrically, the efficacy of EMD associated with BG and an absorbable membrane in the treatment of class III furcation defects. They showed similar results for the experimental groups in all studied parameters and suggested that Emdogain® with bioglass and GTR, or with GTR only, showed similar results when compared with the ones obtained with bioglass associated with membrane in the treatment of class III furcation defects in dogs. The three modalities of treatment showed partial filling of the furcations, with bone and cementum regeneration limited to the apical portion of the defects. Considering from these studies, the combined therapy did not seem to improve the periodontal regeneration.

Results from these animal studies indicate that EMD have the ability to induce the regeneration of periodontal tissues, i.e. cementum, PDL, and bone. The ability of EMD to enhance bone formation has been defined as osteogenic. It enhances the osteoinductive potential of graft materials; thus, osteoinductive material is recommended when bone formation is needed.
1.2.1.3 Clinical Trails

Clinical trials have been conducted for the assessment of the effectiveness of EMD regarding its ability to improve periodontal regeneration and ankylosis prevention. One of the first human studies was a split-mouth randomized multicenter trial undertaken to compare the long-term effect of EMD treatment as an adjunct to MWF surgery vs. MWF plus a placebo (PGA). The results in the EMD group were better, as shown by a gain in the clinical attachment level, probing depth reduction and restoration of bone radiographically.

There are evident from different studies that the use of EMD in periodontal defect appears to be promising. The study of periodontal therapy by means of coronally positioned flaps and EMD was performed with Miller Class I or Class II gingival recession with the two treatments; coronally positioned flap with EMD (test) and coronally positioned flap alone (control). At 12 months, both treatment modalities show significant root coverage, gain in clinical attachment, and gain in width of keratinized gingival. They suggested that addition of EMD with coronally positioned flap significantly improve the amount of root coverage. The other study in treatment alternative to cover exposed root surface is CTG procedure in Miller Class I or Class II buccal recession-type defects. After evaluations, CGT treatment showed a higher percentage of root coverage and increase HKT. They concluded that EMD is a valuable, long-term effective treatment alternative to achieve root coverage together with an increase in HKT. These two studies suggest that EMD-treatment modality show a promising result in gingival defects. In addition, there were several studies that combined EMD with others treatment modalities such as GTR and bioresorbable barrier membrane. The results from these studies revealed an advantage of using EMD in the treatment of periodontal intrabony defects.

There were studies aim to evaluate the effect of EMD in term of ankylosis prevention. The treatment ankylosis of traumatized teeth by intentional replantation using Emdogain® was performed in children. Eleven ankylosed teeth presenting with replacement resorption were treated by tooth extraction, root canal treatment. Emdogain was applied to the root surface and into the extraction socket with subsequent replantation of the tooth. The results from 6 years follow-up period suggest that the treatment of replacement resorption following light to moderate trauma with replantation using Emdogain appears to prevent or delay recurrence of ankylosis. Recently there was a case report of reimplantation of avulsed permanent central and lateral incisor in a 9-year-old girl suffering from a traumatic injury. Emdogain was applied to the root surface and into
the socket with the subsequent replantation of the tooth. Evaluation parameters included horizontal and vertical percussion sound and periapical radiographs. At follow-up period, the clinical and radiographic appearance of the teeth showed resolution of mobility and no signs of replacement resorption. They suggested that using EMD to gain periodontal healing appears to be promising. However in 2005, Andreason et al. studied the effect of Emdogain to traumatized teeth without periodontal tissues in 16 avulsed teeth with varying time of extra oral storage. In this study, PDL cells have been eliminated because of unphysiologic storage or actual damage during avulsion or replantation. After 6 months all teeth showed recurrence of ankylosis. They concluded that Emdogain was not able to prevent or cure ankylosis.

There was a case report described two examples of external inflammatory resorption following surgical root surface debridement and the use of Emdogain® in 2006. Both patients suffered from chronic periodontitis with no other obvious modifying risk factors. The treatment in both cases involved raising a full-thickness flap following completion of non-surgical therapy. The granulation tissue from the defect was removed and the root surfaces debrided. Emdogain® was applied following the manufacturers’ instructions and involved conditioning the root surfaces with Pref-Gel and applying the Emdogain® to the defect. The flaps were sutured and the site reviewed regularly. Radiographs were taken before the treatment was undertaken and also at 6 months to assess the healing of the defect. The results showed that external inflammatory root resorption was observed on the treated teeth 6–24 months after therapy. They concluded that external inflammatory root resorption may be an unusual adverse event following Emdogain® treatment.

Whilst these studies may provide some explanation for the effects of the EMD in ankylosis prevention, and the use of EMD for the treatment of ankylosis appears to be promising, the call for larger controlled clinical trials and the study in clarify the mechanism of this substance are considered necessary.

1.2.2 Bone remodeling

1.2.2.1 Osteoblasts

Osteoblast is epithelial-like cell with only one nucleus an extensive network of rough endoplasmic reticulum, the organelle that is responsible for synthesis of bone matrix proteins. It has two major forms: columnar matrix-producing cell or flattened quiescent cell (sometimes called lining cell). They form a monolayer covering active bone formation area. Osteoblast has
2 main functions, one is to synthesize and deposit the bone matrix during development and throughout life. This function is called bone formation. The second is to control longitudinal bone growth which work together with chondrocytes in cartilage. The osteoblast lineage cells play a central role in the regulation of development and activity of the bone-resorbing osteoclasts. Since it has been shown that osteoblast is the cell type responding to factors that regulate osteoclast formation and activity.

Osteoblasts are derived from multipotent mesenchymal stem cells found in the bone marrow, periosteum and pericytes. The osteoblast precursors reach bone by migration of progenitors from nearby connective tissues. The multipotent mesenchymal stem cells can differentiate into bone marrow stromal cells, chondrocytes, myoblasts, adipocytes, fibroblasts and tendon cells. When the stem cells differentiate into several cell types, they demand the action of cell type-specific factors for development. Two specific factors that modulate differentiation of stem cells to osteoblast are Cbfa1 and osteocalcin. Cbfa1 is an important factor to regulate differentiation of mesenchymal progenitors into the osteoblast lineage. Cbfa1 function in osteoblasts is not only limited to cell differentiation but also regulates the level of bone matrix deposited by differentiated osteoblasts. Osteocalcin is a specific secretor protein expressed only in terminally differentiated osteoblasts under control of Cbfa1.

In embryonal stage, bone tissue is formed through 2 pathways; intramembranous ossification and endochondral ossification. In both pathways, osteoblast plays an important role in the bone formation.

Bone marrow microenvironment produces several growth factors and cytokines such as BMPs, TGF-β, PDGF, IGFs family. They controlled osteoblast differentiation. Other cytokine such as interleukins IL-6 is also capable to control the differentiation of osteoblast.

The sequence of osteoblast differentiation is characterized by the expression of osteoblastic marker which express in each stage of differentiation. When proliferating osteoprogenitors become pre-osteoblasts, cell growth declines, and there is a progressive expression of markers of differentiation by post-mitotic osteoblasts. The sequence of osteogenic differentiation is begin with the expression of ALP, an early marker of osteoblast phenotype, followed by the synthesis and deposition of type 1 collagen, bone matrix proteins, and glycosaminoglycans, and increased in expression of osteocalcin and bone sialoprotein at the onset of mineralization.
The fate of osteoblasts is variable. Human osteoblasts have an average lifespan approximately 1 month. When bone matrix has been deposited and calcified, most osteoblasts decreased matrix synthesis activity and become flattened lining cells. About 10% of osteoblasts are embedded within the matrix synthesized by themselves, and differentiated into osteocytes which connected with each other by cytoplasmic extensions in canaliculi. Osteocytes are smaller than osteoblasts, contain less cell organelles such as ribosomes and endoplasmatic reticula, and have an increased nucleus to cytoplasm ratio. There is a higher number of filopodia, or cytoplasmatic extensions, which serve as interconnection between osteocytes and bone-lining cells, creating a veritable three-dimensional syncitium. This connectivity allows the transfer of molecules and nutrition from the older bone to the bone surface. Nearly half of the osteoblasts appear to undergo apoptosis at the end of bone formation and this fate may contribute to the control of bone formation.

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. Cells are hypo-triploid with a modal chromosome number of 66 and exhibited fibroblast morphology. MG-63 have been well characterized, and widely used in biocompatibility tests. They showed numerous osteoblastic features, including the expression of BMP and osteocalcin. It exhibits 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.

1.2.2.2 Osteoclasts

Osteoclasts are the cell for resorption of the bone tissue on endosteal bone surfaces. They do not belong to the osteoprogenitor cell lineage. They derive from the monocyte-macrophage progenitor cell lineage in the bone marrow, which diverge into the osteoclast progenitor pathway. The osteoclast progenitor cells are monocytes, which reach the bone through the blood circulation and fuse into multinucleated cells with as many as 30 nuclei to form osteoclasts by a process regulated by osteoblasts and stromal cells of the bone marrow.

Bone resorbing cells are large multinucleated (up to 100 µM), highly migratory, non-dividing giant cell with a relatively short lifespan. They are usually extremely rare in bone with only 2-3 cells/mm. An activated osteoclasts are able to resorb 200,000 µm³/day, an amount of bone formed by seven to ten generations of osteoblasts with an average lifespan of 15-20 days. Osteoclasts possess numerous mitochondria, the source of ATP to drive the H⁺ pumps required
for the acidification of the subosteoclastic compartment, and an extensive Golgi system but have a sparse endoplasmic reticulum and few ribosomes. 

Osteoclasts lack many of the antigens that are characteristic of macrophages and express very high levels of TRAP and VNR; they also express CTRs that are absent from macrophages. These features are commonly used for the detection of osteoclasts in bone specimens.

Osteoclast activity is directly regulated by calcitonin (synthesized by neural crest derived parafollicular or C cells of the thyroid follicle), vitamin D₃, and regulatory molecules produced by osteoblasts and stromal cells of the bone marrow. There are various enzymes producing by osteoclasts, TRAP is quite specific. However its role in bone resorption is not clear. Osteoclasts also produce enzymes, such as cathepsins, which are able to degrade collagen at the low pH in the resorption microcompartment. Osteoclasts produce cathepsin B, D, and L. Recently cathepsin K is known to be the main enzyme responsible for the degradation of the bone matrix. Knock-out of cathepsin K induces inhibition of bone resorption and osteopetrosis in mice. They also produce a large numbers of vitronectin receptors. Osteoclast life span is not known in humans and they undergo apoptosis like any cell.

When resorbing bone, osteoclasts are tightly attached to the bone surface and create “‘sealing zone’” an area of the cells containing large amounts of F-actin fibers perpendicular to the bone surface, and they create several other functional domains within their plasma membrane. The area directly opposite the bone surface will develop many folds and ruffles and is called the “‘ruffled border’. This area of plasma membrane contains a high density of vitronectin and collagen receptors and also contains a proton pump which secretes acid into the resorption cavity to dissolve the bone leaving pit in the bone surface.

1.2.2.3 Osteoclastogenesis

After the proliferation of osteoclast progenitors cell, mononuclear osteoclast precursor cells are recruited from the bone marrow, or other hematopoietic sites via the circulation. The precursors are guided to sites where resorption of the bone tissue is occur. The mechanism and cell type responsible for the recruitment of precursors are still unknown. Osteoclast precursor cells are differentiate into mononuclear prefusion osteoclasts. Then they are fusion results in formation of mature osteoclasts, clear zone (actin ring) and ruffled border formation (activation), and finally apoptosis. There are several factors that control osteoclastogenesis. The cytokines synthesizing
by bone marrow stromal cells and osteoblasts such as M-CSF, RANKL, OPG and TNF-α. M-CSF is a secretary protein of osteoblasts and their precursors. Binding of M-CSF to its receptor c-Fms on osteoclast progenitor cells promotes proliferation and survival of these cells. Cell-to-cell contact with cells from the osteoblastic lineage is necessary for differentiation and activation of the osteoclast precursors. This is mediated by binding of RANKL, producing by osteoblasts and stromal cells. It is an anti-apoptotic factor and an activation factor for osteoclast. RANKL binds to its receptor, RANK on osteoclast precursors and multinuclear osteoclasts. The RANKL-RANK interaction promotes differentiation of the osteoclast progenitor cells, fusion of preosteoclasts and activation of multinucleated osteoclasts, resulting in development of multinucleated, finally differentiated osteoclasts that are able to resorbed bone.

The osteoblasts and activated T cells produce OPG, a soluble glycoprotein with the ability as a decoy receptor in the RANKL-RANK interaction, resulting in inhibition of osteoclastic development and subsequent bone resorption. To date OPG is a powerful inhibitor of osteoclast formation both in vivo and in vitro model.

The importance growth factor in bone matrix that has effect on bone resorbing cells is TGF-β. Its effect is directly inducing mononuclear phagocyte osteoclast precursors to differentiate into osteoclastic cells capable of bone resorption.

Osteoclasts and osteoblasts are tightly regulated in order to maintain a dynamic equilibrium between their formation, survival, and function. Many critical molecules appear to exert both stimulatory and inhibitory effects, both directly and indirectly, upon both the bone forming and bone resorbing cells as following.

The two principal hormones of the calcium homeostatic system, namely Parathyroid hormones and 1, 25- (OH)₂D₃ stimulate osteoclast formation. Calcitonin, the bone-regulating hormones, inhibits osteoclast development and activity and promotes osteoclast apoptosis. Several other hormones, including estrogen, androgen and glucocorticoids are potent regulatory influences on the development of osteoclasts and osteoblasts by regulating the production and/or action of several cytokines.

Bisphosphonates, a stable carbon-substituted pyrophosphate analogue, are proven to be an effective inhibitor of osteolysis not only by inhibit osteoclastic cell recruitment but also induce osteoclast and macrophage apoptosis.
1.2.2.4 Crosstalk between osteoblasts and osteoclasts

Osteoblasts and osteoclasts are locked in tightly regulated in order to maintain the equilibrium between their formation, survival and function. The intercellular communication between them is crucial for bone formation and bone resorption. Various molecules appear to exert both stimulatory and inhibitory effects. In normal conditions, amounts of bone formation are equal to bone resorption. Osteoclasts differentiation, formation and activation are depend upon the proximity and products from the osteoblasts and while osteoclasts are resorbing bone, there are growth factors that releasing from this process to effect the osteoblast differentiation.

Osteoclasts are multinucleated giants cells that derived from haematopoietic stem cells. The osteoclast precursors proliferation are stimulated to maintain a pool of mononuclear cells in monocyte/macrophage lineage by M-CSF. M-CSF is a critical early modulator in differentiation of osteoclasts by enhancing the survival of monocytic stem cells. It is a soluble factor produced by osteoblasts/stromal cells. It promotes the proliferation, survival, and differentiation of monocytes and macrophages and plays an important role in the survival and proliferation of osteoclasts progenitors cells. M-CSF binding to its receptor c-fms, a receptor tyrosine kinase on the cell surface at many stage of osteoclasts development results in promotion of cell survival and proliferation. Then the mononuclear precursors are attracted to the area of further resorption site and differentiate into prefusion osteoclasts with the stimulation of M-CSF and RANKL. RANKL is a novel member of the TNF superfamily. RANKL is produced in various tissues and cell types especially in bone and lymphoid tissues and binds to the RANK receptor. It is present on the surface of osteoclastic precursors, thereby stimulating the differentiation and activation of osteoclasts. RANKL is essential and sufficient for all stage of osteoclast differentiation. RANK is expressed by late stage of osteoclastic precursors cells. The decoy receptor for RANKL is OPG, the secreted member of TNFR superfamily. The biological effects of OPG include inhibition of proliferation, differentiation, survival and fusion of osteoclastic precursor cells; inhibition of the activation of osteoclasts; and promotion of the osteoclast apoptosis. Mice with ablation OPG gene showed severe early osteoporosis due to excessive bone resorption.

Osteoclasts also regulate osteoblast differentiation by several secreted molecules. There are several growth factors released from bone matrix during bone resorption that can control osteoblastogenesis. TGF-β is the growth factors and specific components in the bone matrix. It is
released by osteoclasts during bone resorption. TGF-β produced by many cells in the bone microenvironment; osteoblasts, fibroblasts and osteoclasts. TGF-β receptors are expressed in osteoblasts and mononuclear phagocyte osteoclast precursors. Therefore, these cells are known to be influenced by TGF-β. The effect of TGF-β is a bi-directional manner depend on the stage of maturation of the osteoblasts. TGF-β can both stimulate of osteoblast recruitment, migration, proliferation of osteoblast precursors and inhibition of terminal osteoblastic differentiation into active osteoblasts. TGF-β increase osteoblast and bone marrow stromal cell production of OPG and decrease the expression of RANKL. It can both enhance and inhibit osteoclast formation and resorption, depending on the assay system used to measure these parameters.

In addition, osteoclasts produce and secrete BMP-2. It is belongs to TGF-β superfamily, member of which is known to regulate the proliferation, differentiation and death of cells in various tissues. Similar to TGF-β, BMP-2 appears to regulate directly in osteoclasts to potentates RANKL mediate formation and survival.

During osteoclasts resorbed bone matrix, they also release several growth factors that stimulate osteoblast proliferation, including IGF-1 and II, FGFS and PDGF. These growth factors are reported to prevent osteoblast apoptosis in vitro. Furthermore, osteoclasts secrete Mim-1; an osteoclast secreted protein. Mim-1 is expressed by cells of haematopoietic origins. Mim-1 can stimulates migration and differentiation of osteoblastics precursor cells.

In summary, osteoblasts and osteoclasts are responding to numerous factors that are derived from bone or are in the bone microenvironment. These cells have intercommunication signaling to regulate each other. Under normal conditions, they are balance between their formation, survival and function leading to normal bone remodeling (bone formation equal bone resorption). The crosstalk between osteoblasts and osteoclasts also influenced by other factor such as aging, hormones, mechanical forces and other diseases.

### 1.2.2.5 Bone Remodeling

Bone is a specialized connective tissue formed by a mineralized matrix by the bone forming cell; osteoblast. This tissue has three functions; mechanical, protective for the vital organ, and metabolic as a reservoir of ions especially calcium and phosphorus (about 80% of bone matrix is calcium hydroxyapatite). The two types of bone are cortical and trabecular bone. Cortical bone is a layer that forms the outer shell of bone and compose of thick, dense calcify tissues. The
trabecular bone forms an interior meshwork of bone. There are three cell types found within bone tissue; osteoblasts, osteoclasts and osteocytes. In the skeletal, osteoblasts and osteoclasts belong to the temporally structure, known as BMU. The size of BMU is approximately 1-2 mm long and 0.2-0.4 mm wide. The life span is 6-9 months. BMU is comprise of osteoclasts, osteoblasts, vascular capillary, nerve supply and associated connective tissue. Bone tissue undergoes continual resorption of old bone and replacement with the exactly the same amount of newly bone in order to maintain the bone structure; bone integrity and calcium homeostasis throughout life. This process is called bone remodeling. In human, after 1 year of age, bone start to remodeling. It is a complex process performed by complex communication between osteoblasts and osteoclasts.

In normal condition, the activity of osteoblasts and osteoclasts is balanced, so the net result of this process is zero. A disturbance of osteoblast or osteoclast function can have the effects in bone remodeling process leading to common bone disease such as osteoporosis, osteopetrosis, periodontitis, arthritis and tumors-induced osteolysis.

Bone formation throughout life is a function of the bone-forming cell, osteoblast. The rate of bone formation is dependent on the process of osteoblastogenesis and the lifespan of osteoblasts. In adult human bone remodeling cycle take approximately three months. This events and signaling behind this process is still unknown. Bone surfaces are covered with osteoblastic cells, which are inactivated state when bone remodeling does not occur (quiescent phrase). The process takes place in multiple locations in the skeletal at the same time. After the activation of quiescent osteoblast, which regulated by several factors including both systemic hormones and local factors. The activated osteoblasts begin to degrade the osteoid, the non-mineralized matrix to enable for osteoclasts to attract and start to resorb bone matrix. After that osteoclast are attracted to the resorptive area via the circulation and then start to resorb bone until lacunae is about 100 µm in diameter and 50 µm deep which take about 10 days to finish. The resorption process results in releasing of mineralized matrix of non-collageneous proteins, which deposited by osteoblasts during bone formation. When osteoclasts have stopped and detached from the bone lacunae, the osteoblasts near the resorbed area are stimulated with the growth factors that release from resorption process. Osteoblasts begin to form bone at the bottom of the lacunae and lay down the osteoid matrix. When the lacuna is fulfill with osteoid, this process take about 80 days. Then mineralized with hydroxyapatite occurs. The remodeled area then passes into the quiescent state. Complete this remodeling process takes about 60-120 days.
Systemic hormones and local factors which effect both of osteoblast and osteoclasts are also control bone remodeling. Hormones that regulate bone remodeling include parathyroid hormone, calcitonin, insulin, growth hormone, steroids and thyroid hormones. The growth factors such as IGF I and II, TGF-β superfamily, fibroblasts growth factors are also play a critical role in bone remodeling and in the pathophysiology of bone disease.\textsuperscript{127}

\section*{1.2.2.6 OPG/RANKL/RANK}

\textbf{OPG}

OPG was the first of the three molecules to be identified. Osteoclastogenesis inhibitory factor (OCIF) is the first name of OPG given by Tsuda and co-workers in 1997. This protein are secreted from human fibroblasts that able to inhibit bone resorption.\textsuperscript{116} At the same time, Simonet and colleagues reported a protein cloned from a rat intestinal cDNA with actions similar to those of OCIF, which was termed osteoprotegerin (OPG).\textsuperscript{96} Simultaneously, Tan et al. found the protein that inhibit osteoclasts formation in a sequence tag database and termed TR1 for its similarity to the members of the TNF receptor superfamily.\textsuperscript{131} OCIF and TR-1 were later found to be identical to OPG.\textsuperscript{115,132} In addition, Yun and colleagues discovered a TNF-related receptor in lymphoid cells that was named follicular dendritic cell-derived receptor-1 (FDCR-1), which also was identical to OPG.\textsuperscript{133}

OPG gene is consists of 401 amino acid propeptide, which is cleaved to a biologically active 380 amino acid protein that can exist as both monomer and disulfide-linked homodimer. Human, mouse, and rat OPG share 85-94\% sequence rich domains in the N-terminal of the OPG protein.\textsuperscript{96,115} OPG lacks transmembrane domain and cytoplasmic tail and consequently exists as a soluble receptor.\textsuperscript{96,115}

OPG mRNA expression have been found in several organs and tissues, including cartilage, bone, stomach, intestine, skin, lung, liver, heart, kidney, brain and lymphoid cells.\textsuperscript{134} The importance of OPG in development of osteoclasts and bone resorption has been studied using both OPG-deficient mice and transgenic mice overexpressing OPG. The opg-/- knockout mice develop severe early-onset osteoporosis due to excessive osteoclast formation and activity.\textsuperscript{117,135} The targeted deletion of OPG also caused severe hypercalcemia.\textsuperscript{117} Meanwhile, overexpression of OPG in mice results in osteopetrosis with increased bone mineral density and decreased osteoclast
formation. It can suggest that OPG are therapeutic target in treatment of increased bone resorption in pathological conditions such as osteoporosis and rheumatoid arthritis.

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. The ratio of RANKL to OPG levels has been shown to be important in bone formation or bone resorption; the balance of this ratio is important in bone homeostasis, an increased ratio is associated with a higher risk for osteolytic lesions and mortality.

**RANKL**

RANKL is a type II transmembrane protein which has been shown to be expressed on the cell surface of activated T cells. It is belongs to TNF ligand superfamily that has two active forms; membrane-bound (40-45 kDa) and soluble forms (31 kDa). Human and mouse RANKL are 317 and 316 amino acid peptides, respectively. RANKL mRNA is highly expressed in bone, bone marrow and in lymphoid tissue including fetal liver, lymphnodes and thymus. Low levels of RANKL mRNA can be detected in spleen, bone marrow, peripheral blood, leukocytes, heart, placenta, skeletal muscle, stomach and the thyroid. Its expression are upregulated by glucocorticoids, 1,25dihydroxyvitamin D3, IL-1, IL-6, IL-11, IL-17, TNF-α, PGE2, or PTH. RANKL is necessary for osteoclast development, activation of mature osteoclast and decrease osteoclast apoptosis. RANKL over expression has been shown in patients with malignant osteolysis due to myeloma or bone metastases, in the rheumatoid synovial pannus, and in rats with osteoporosis due to ovariectomy.

RANKL binds to its receptor RANK through the extracellular domain consisting of four receptor-binding loops. Both 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. RANKL action is negatively regulated by OPG which antagonizing binding to RANK.

**RANK**

RANK is a type I transmembrane protein of 616 amino acids that belongs to subfamily of TNFR superfamily. RANK is expressed in skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, osteoclast, mammary gland epithelial cells, prostate and pancreas.
RANK expression in bone cell act as a marker of the osteoclastic phenotype. Stimulating RANK promotes osteoclastogenesis, whereas inhibitory RANK results in suppressed osteoclastogenesis. The binding of RANKL to RANK is an important role in promoting osteoclast differentiation and bone resorption. Mice with mutation of RANK display severe osteopetrosis, growth retardation, and a defect in tooth eruption. Transgenic mice that overexpress the soluble RANK and mice with targeted deletion of the normal RANK were reported to display severe osteopetrosis due to a lack of mature osteoclasts, indicating that RANK is requirement for normal osteoclast development. RANKL/RANK system is implicated in the pathogenesis of bone disease such as postmenopausal osteoporosis, bone loss in rheumatoid arthritis and osteolysis tumor.

1.3 Aims of the study

The aims of this study were

1. To determine the expression of OPG and RANKL mRNA in osteoblast-like cell MG-63 in the presence or absence of Emdogain.

2. To examine the effects of Emdogain on osteoclast.