

# In vitro studies of Enamel Matrix Derivative In Terms of Periodontal Wound Healing and Periodontal Regeneration

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## Abstract

Periodontal disease results in the loss of attachment apparatus. An increasing effort has been placed on seeking procedures and materials to promote the regeneration of this tissue. An extract of embryonic enamel matrix, termed 'Enamel Matrix Derivative' (EMD), is thought to induce mesenchymal cells to mimic the processes that take place during the development of the root and periodontal tissues, suggesting a promising material which can push the periodontal regeneration cascade forwards by means of several suggested mechanisms. The aim of this paper is to deepen the reader's knowledge of the in vitro effects of EMD in terms of periodontal wound healing and periodontal regeneration. Different features of EMD, the effect of EMD on the cells that are related to periodontal regeneration and the effect of EMD on other cell types are demonstrated in this review. It was concluded that EMD regulates multiple cell types and enhances several cellular mechanisms of periodontal ligament cells. In contrast to its effects on mesenchymal cells, EMD appears to inhibit the proliferation and growth of epithelial cells. The greatest effect of EMD has been shown to be on mature cells, suggesting that it may not be capable of controlling the entire process of periodontal regeneration.

## INTRODUCTION

In 1997, new approach for periodontal regeneration was introduced that was based on embryonic tooth formation (1,2). This approach uses an extract of embryonic enamel matrix, termed 'Enamel Matrix Derivative' (EMD), thought to induce mesenchymal cell to mimic the processes that take place during the development of the nascent root and periodontal tissues.

The enamel matrix was generally believed to regulate the initiation, propagation, termination, and maturation of the enamel hydroxyapatite crystallites (3). Other findings indicate that the enamel matrix also has a function outside the developing enamel. Enamel matrix proteins are temporarily deposited onto the dentinal root surface and provide an initial and essential step in the formation of acellular cementum (4,5). Autoradiographic and scanning electron microscopy studies provide additional evidence that, following apoptosis of Hertwig's epithelial root sheath (HERS) cells and deposition of the enamel matrix proteins onto the dentine surface, the cementogenesis process is initiated and kept modulated by these proteins (6,7,8). Subsequently, when cementum has been laid down onto the enamel-matrix-covered dentin surface, an attachment apparatus will develop. Immunological (9) and

immunohistochemical (1) methods both show that enamel matrix proteins are present in acellular cementum, accentuating the importance of these proteins in the cementogenesis process.

## COMPOSITION OF THE ENAMEL MATRIX DERIVATIVES

The major fraction of the enamel matrix derivatives is composed of the amelogenins, a family of hydrophobic proteins that account for more than 90% of the organic constituent of the enamel matrix (10). The amelogenins have remained remarkably well-conserved through evolution, suggesting that they may have great functional importance.

The second largest component of the enamel matrix proteins is the enamelines (10). Since the enamelines were found to contain serum proteins (11,12), the more general term "non-amelogenin" is now commonly used to describe this high-molecular-weight fraction (1). It includes praline-rich enamelin (13), tuftelin (14), and tuft proteins (15).

Three matrix proteins, corresponding to amelogenin (16), enamelin (17), and sheathlin (also called ameloblastin or amelin) (18), and 2 enzymes, corresponding to MMP-20 (19) and EMSP1 (20), have been purified and the cDNA cloned

from developing porcine teeth. These proteins are all present in EMD. Although early immunoassay studies could not identify the presence of the growth factors in EMD <sup>(21)</sup>, nominal levels of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) have been detected immunologically <sup>(22)</sup>. In addition, by using the bone morphogenetic protein (BMP) binding protein noggin, investigators have identified BMP-2 and BMP-4 in an osteoinductive fraction of enamel extract <sup>(23)</sup>. Even though the latter study used non-commercial fractionated enamel extracts from developing pig teeth, it may suggest the presence of these morphogenetic proteins in commercial EMD as well.

### **EMDOGAIN® FORMULATION**

A commercial 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-month-old piglets. 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 <sup>(1,2)</sup>. The enamel proteins described above are present in Emdogain®.

The amelogenins, which are the hydrophobic constituent of the enamel matrix proteins, aggregate and become practically insoluble at physiologic pH and body temperature. They can be dissolved in an acidic or alkaline pH environment and at low temperature. A suitable formulation of EMD should thus have a non-neutral pH and allow for gradual re-precipitation of the matrix when physiological conditions are re-established. In order to create a suitable vehicle for Emdogain®, many studies showed that propylene glycol alginate (PGA) was more effective than hydroxyethyl cellulose (HEC) or dextran. PGA appears to enhance EMD precipitation, thus exposing the periodontal ligament cells to the re-established protein aggregate and allowing the (matrix:cell) interaction to take place. The other vehicles that were tested, which were stable at neutral pH, appear to exclude the periodontal ligament cells from exposure to the proteins <sup>(1)</sup>.

Since the commercial formulation of EMD (Emdogain®) is a porcine-derived material (i.e., a xenograft), the potential for it to stimulate an immune reaction when used in humans is of extreme importance.

In vitro studies showed that EMD does not significantly modify cellular or humoral immune responses. Very high

concentrations of EMD induced only a slight increase in the proliferation of human lymphocytes, restricted to the CD25+ (IL-2 receptor) fraction of the CD4+ T-lymphocytes. There was a concomitant decrease of B-lymphocytes, while other cell fractions (CD8+ T-cells, B-cells, and NK cells) were not affected, and immunoglobulin and cytokine (IL-2 and IL-6) production was not modified <sup>(24)</sup>.

### **ENAMEL MATRIX DERIVATIVE MECHANISMS IN TERMS OF PERIODONTAL WOUND HEALING AND PERIODONTAL REGENERATION**

Periodontal wound healing and regeneration involve the recruitment of connective tissue cells to the wound site, proliferation of the cells, and subsequent organization of mature tissue structure. The initial events of new matrix synthesis occurring during wound healing appear to be the production of hyaluronan (HA), followed by proteoglycan synthesis prior to collagen deposition. During these events, the cells must interact with a variety of soluble mediator which directs the course of remodeling through a combination of cell-cell and cell-matrix interaction.

Concerning these data, it has been shown that EMD could stimulate matrix synthesis in vitro through modulation of hyaluronan and proteoglycan synthesis in a manner consistent with changes noted in tissues undergoing repair and regeneration <sup>(25)</sup>. However, it has been shown that EMD – when acting under organoid culture - did not promote mineralization <sup>(26)</sup>.

Bosshardt et al. <sup>(27)</sup> conducted a study to characterize the nature of the tissue that forms on the root surface following application of EMD. The newly formed tissues on the root had been shown to be thick and contained embedded cells. Small mineralization foci were regularly seen, and large organic matrix patches were occasionally seen, but a distinct mineralization front was lacking. It was concluded that tissues resembling either cellular intrinsic fiber cementum or a type of bone were observed. The mineralization pattern mostly resembled that found in bone, except for a few areas that exhibited a hitherto undescribed mineralization pattern.

Many of the processes which take place during Periodontal wound healing are controlled by cytokines and growth factors.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) plays a role in the process of wound healing <sup>(28)</sup> and is a potent regulator of integrin expression <sup>(29)</sup>. This growth factor might play some

role in stimulating periodontal healing and regeneration (30).

While Emdogain® (EMD) is not strictly considered a growth factor, it appears to possess a number of properties that are 'growth factor-like'. It has been suggested that enamel extract have bone morphogenetic protein BMP-like activity (23) and (TGF- $\beta$ )-like activity (22), Suzuki et al. (31) hypothesized that BMP and TGF- $\beta$  may be involved in fractionated EMD-Gel by means of luciferase reporter assay as they could show that EMD-Gel contained BMP-like growth factor from fraction 4 to 6, and these fraction contained proteins of 20 kDa, similar to BMP (32).

Moreover, it has been shown that EMD-Gel contains TGF- $\beta$ -like growth factor that contributes to the induction of biomineralization during periodontal regeneration.

Nagano et al. (33) suggested through an in vitro study that fractionated porcine enamel proteins contain both BMPs and TGF- $\beta$ 1. They concluded that the enamel matrix of developing teeth contains TGF- $\beta$ 1, which may interact with BMPs and contribute to the differentiation of periodontal mesenchymal cells, and the osteoinductive activity of EMD on human periodontal ligament (HPDL) cells is mediated by TGF- $\beta$ 1.

Undoubtedly the key for achieving complete periodontal regeneration lies in a better and more comprehensive understanding of the cellular and molecular mechanisms which underlie the wound healing and regenerative process. In this context, the study of Parkar and Tonetti (34) was undertaken to examine the effects of EMD on the synthesis of growth factors, cytokines, and their receptors by periodontal ligament (PDL) cells using a selective cDNA array comprising 268 genes of particular relevance to the wound-healing process, and It has been shown that EMD down-regulate the expression of genes involved in the early inflammatory phase of wound healing while simultaneously upregulating genes encoding growth and repair-promoting molecules. This may partly explain the apparent efficacy of EMD application in periodontal regeneration.

Recently, new light has been shed on the complex mechanisms underlying bone remodeling in terms of periodontal regeneration. Osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) have been shown to play a key role in regulating this balance, as RANKL binds its receptor (RANK) on the osteoclast precursor surface, determining their activation and

differentiation to mature osteoclasts (35).

RANKL, a member of the tumor necrotizing factor TNF, is expressed by cells of the osteoblastic lineage, including stromal cells, and by circulating T lymphocytes, as a membrane-bound or soluble form (36,37). RANKL signaling can be blocked upon binding to the decoy receptor, OPG, which occupies RANKL binding sites and protects bone from resorption.

OPG exerts its effect by acting as a RANKL decoy receptor, thus preventing its binding to osteoclasts and inhibiting their activation (38). These molecules are the most powerful osteoclast regulators, and so they can be reasonably considered key targets of any bioactive factor-based bone engineering technique aiming to shift the bone formation/resorption balance toward tissue regeneration.

Although it has recently been proven that EMD enhances OPG expression by MC3T3 cells (39), its influence on OPG and RANKL synthesis in human alveolar bone cells still has to be determined.

In the same field, Galli et al. (40) investigated EMD's effects on human primary mandibular osteoblasts, their differentiation, and the expression of factors regulating bone balance, and, therefore, a potential rationale for EMD use as an adjunctive bone-stimulating factor. It has been shown that EMD was able to enhance osteoblast cell growth and the expression of markers of osteoblastic phenotype and differentiation. EMD also seemed able to create a favorable osteogenic microenvironment by reducing RANKL release and enhancing osteoblastic OPG production.

On the other hand, EMD has been found to induce the formation of osteoclasts through interaction with RANKL (41). The role of bone metabolism molecular regulators, including OPG, RANKL, Cyclooxygenase 2 (COX2) and core binding factor alpha 1 (Cbfal), within the context of EMD stimulation of mineralization tissue formation is still not clear. Therefore, Takayanagi et al. (42) conducted a study to explore the effect of EMD on these bone-related molecules in human periodontal ligament PDL cells. The results of this study have shown that there is a significant increase in COX2 mRNA levels with EMD treatment, and no effects were noted on mRNA levels for Cbfal. RANKL mRNA levels were significantly decreased with EMD treatment. OPG levels showed minimal effects with EMD treatment. However, the RANKL/OPG ration showed a 40%

to 55% reduction with EMD  $\leq 25$  microg/ml. These results support a role for EMD stimulation of mineralized tissue formation consistent with periodontal regeneration by modulating regulatory molecules critical to bone metabolism at the RNA level.

New blood vessel formation (angiogenesis), the growth of new blood vessels from existing ones, is important in a number of normal physiologic settings, including growth, development, reproduction, and wound healing<sup>(43)</sup>. Angiogenesis is one of the most critical events in the periodontal wound healing process. Any increase in angiogenesis could result in more rapid and complete healing. Subsequent to an injury, capillaries invading a fibrin clot are very important in early granulation tissue formation because they deliver nutrients, inflammatory cells, and oxygen to the wound site<sup>(44)</sup>. On the contrary, a lack of angiogenesis can result in impaired and delayed wound healing and, usually, ulcer formation<sup>(45,46)</sup>.

Yuan et al.<sup>(47)</sup> found that EMD had an in vitro angiogenic effect, and that EMD exhibits a chemotactic effect, but not a proliferative effect, on endothelial cells. The hydrophobic proteins content of EMD precipitate in neutral pH conditions; therefore, it is very unlikely that they contributed directly to the angiogenic effect mentioned in this study. Bellahcene et al.<sup>(48)</sup> revealed that bone sialoprotein (BSP) was able to promote angiogenesis through its cognate receptor, integrin  $\alpha_v\beta_3$ . Therefore, the chemotactic effect of endothelial cells to the EMD product could be partially explained due to the existence of BSP-like protein.

However, the clinical finding of faster initial healing after the application of EMD gel was not the result of the angiogenic effect of EMD alone. Other mechanisms related to TGF- $\beta$ 1 and platelet-derived growth factor (PDGF) secretion were suggested.

In the same field, it has been shown that EMD stimulates angiogenesis directly by stimulating endothelial cells and indirectly by stimulating the production of vascular endothelial growth factor (VEGF) by PDL cells<sup>(49)</sup>.

Importantly, the data are consistent with the concept that EMD enhances bidirectional communication between human microvascular endothelial cells (HMVEC) and PDL cells during angiogenesis associated with healing.

## **THE EFFECT OF EMD ON PDL CELLS AND**

## **GINGIVAL FIBROBLASTS**

The mode of action of EMD appears to be through a matrix-cell interaction between the amelogenin aggregate of the EMD and the cells of periodontal ligament<sup>(1,2,50,51,52)</sup>. Interestingly, enhanced soft tissues wound healing has been noted following the use of EMD<sup>(53)</sup>.

In order to understand the EMD's mode of action during the periodontal regeneration, many in vitro studies had been established concerning the effects of EMD on PDL cells.

The study of Gestrelus et al.<sup>(21)</sup> showed significant stimulation of cellular proliferation and protein and collagen production and formation of mineralized nodules in PDL cell cultures exposed to EMD. In contrast, addition of EMD had no significant effect on epithelial cell proliferation<sup>(21,54)</sup>.

Van der Pauw et al.<sup>(55)</sup> investigated the effects of EMD proteins on the behavior of human periodontal ligament fibroblasts (HPLF) and human gingival fibroblast (HGF) in vitro and it has been shown that HGF barely attached and spread on EMD-coated substrata, whereas HPLF attached and spread within 24 hours. Further more, the expression of alkaline phosphatase (ALP) activity was significantly enhanced under the influence of EMDs, especially in HPLF. HPLF and HGF both released significantly higher levels of TGF- $\beta$ 1 in the presence of EMD.

Using an in vitro system to evaluate wound-fill rates, EMD has been shown to be a significant stimulator of human PDL cells<sup>(56)</sup>. This effect by EMD was greater than the effect of platelet-derived growth factor PDGF-BB, a proliferation agent of human PDL cells. It was also shown that EMD stimulated wound-fill rates of gingival fibroblasts cells.

Chen et al.<sup>(57)</sup> introduced enamel matrix proteins loaded dextran-based hydrogel microspheres (EMPs-dex-MPs) and could show that this complex was homogenous and stable and enhanced the proliferation response and ALP activities of human periodontal ligament cells.

Lossoerfer et al.<sup>(58)</sup> conducted a study hypothesized that combined exposure to EMD and parathyroid hormone (PTH), which acts anabolically when administered intermittently, would enhance periodontal ligament cell proliferation and differentiation. However, the results of this study suggested that EMD promotes periodontal ligament cell differentiation and osteoprotegerin production, potentially resulting in a microenvironment supporting

periodontal repair, whereas combining EMD and PTH failed to prove beneficial in this respect.

Rincon et al. <sup>(59)</sup> studied the effect of EMD on proliferation of gingival fibroblasts and periodontal ligament fibroblasts in an in vitro wound-healing model, and it has been shown that the cells fill an empty space by a combination of proliferation and cell migration. The most rapid closure of a wound area occurred where both proliferation and migration can occur as was seen when wounded culture were maintained in 10% fetal calf serum (FCS) or at a concentration of 20 µg/ml EMD which promoted proliferation. Therefore, EMD appears to exert an influence on periodontal cells that is compatible with improved wound healing.

Lyngstadaas et al. <sup>(51)</sup> investigated the effect of EMD on a culture of PDL cells and epithelial cells. Cultured PDL cells exposed to EMD increase attachment rate, growth rate and metabolism, and subsequently release several growth factors into the medium. The cellular interaction with EMD generate an intercellular cyclic-AMP (cAMP) signal, after which cells secrete TGF- $\beta$ 1, interleukin-6 (IL-6) and PDGF-AB. Epithelial cell growth however, is inhibited by the same signal. This suggest that EMD favors mesenchymal cell growth over epithelium, and that autocrine growth factors released by PDL cells exposed to EMD contribute to periodontal healing and regeneration in a process mimicking natural root development.

Okubo et al. <sup>(60)</sup> reported that EMD had no effect on osteoblastic differentiation of PDL cells and that EMD is not osteoinductive on HGF but that PDL fibroblasts may respond to EMD differently from HGF. It has also been shown that EMD increased the cell number of gingival fibroblasts as well as the amount of matrix produced. This observation could suggest the possible use of EMD in tissue engineering of gingival and connective tissue grafts for root coverage procedure and could also explain the observation of increased width of keratinized tissue following a coronally positioned flap procedure <sup>(61)</sup> and faster healing of gingival wound <sup>(53)</sup> when EMD was applied. In another study of Keila et al. <sup>(62)</sup>, no mineralization occurred when gingival fibroblasts were cultured in the presence of 25 or 100 µg/mL EMD for a period of 4 weeks. In support, ALP activity of these cells was negligible. Thus, EMD is not able to induce osteoblastic differentiation of GF, analysis of these data fits well with the notion that EMD is not osteoinductive <sup>(63)</sup>. However, these data differ from a previous report where

EMD slightly induced ALP activity in these cells <sup>(55)</sup>

To evaluate the ability of EMD to regulate osteoblast proliferation, differentiation, and to determine if this effect is dependent on the stage of cell maturation, Schwartz et al. <sup>(64)</sup> used cell culture models at different states of maturation in the osteoblastic lineage; 2T9 cells (pre-osteoblasts), MG63 (immature osteoblasts), and normal human osteoblast-like cells (mature osteoblasts). It was shown that EMD affects early states of osteoblastic maturation by stimulating proliferation, but as cells mature in the lineage, EMD enhance differentiation.

Yoneda et al. <sup>(65)</sup> used two mouse osteoblastic cell lines and showed that the effect of EMD was cell-type dependent and that the overall effect of EMD on osteoblastic cells is stimulatory rather than inhibitory. Other studies also observed different responses of the osteoblastic cell lines with EMD <sup>(64,66)</sup>.

Carinci et al. <sup>(67)</sup> attempted to find out how EMD acts on osteoblasts using a microarray technique to identify genes that are differently regulated in osteoblasts exposed to EMD, and it has been shown that the differentially expressed genes cover a broad range of functional activities: (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.

While Van der Pauw et al. reported that EMD stimulate cellular attachment of (HPDL) cells in vitro <sup>(55)</sup>, it was not clear which components of EMD were responsible for the attachment effects observed.

Suzuki et al. <sup>(68)</sup> evaluated which molecules in EMD mediate cellular attachment, and it was shown that the interaction between BSP-like molecules in EMD and integrin  $\alpha$ v $\beta$ 3 on the cell surface were responsible for the cellular attachment mentioned above. This study appeared to be the first to suggest that EMD contains a 55 kDa BSP-like molecule, and it had shown that interaction between BSP-like molecule in EMD and integrin  $\alpha$ v $\beta$ 3 on the HPDL cell's surface might be critical not only for cell attachment but also for differentiation of HPDL cells.

PDL fibroblasts have the capacity to regenerate a periodontal defect with cementum, bone, and periodontal ligament by differentiating into cells of cementogenic, osteogenic, and

fibroblastic lineage (69,70).

Carnes et al. (71) could isolate fibroblasts with osteogenic characteristics compared to cells with fibroblastic characteristics using the expression of alkaline phosphatase activity and osteocalcin release. Fibroblasts that produced high alkaline phosphatase typically formed mineralized matrix. Thus, different phenotypes of PDL fibroblasts can be isolated and may respond differently to attempts at regeneration through biomimetics and may explain the seemingly contradictory results in published studies regarding the in vitro effects of EMD.

Chong et al. (72) used two different cell types of fibroblasts to examine their response to EMD, PDGF-BB and amelogenin. The results validated the clinical effect of EMD by suggesting several possible mechanisms; First, EMD by itself has limited effects on PDL fibroblast proliferation. However, EMD can enhance the proliferative effects of PDGF-BB on ALP (+) fibroblast lineage. Second, EMD enhances wound fill rates in ALP (+) and ALP(-) fibroblasts, primarily near the wound edge, and additively with PDGF-BB at the center of the wound. Third, the regenerative effects of EMD cannot be attributed to amelogenin alone. The results of this study propose several new concepts of EMD and its effects on periodontal regeneration. According to Hoang et al. (56), amelogenin in EMD promotes cell adhesion activity. Because amelogenin binds to hydroxyapatite, EMD may enhance attachment of fibroblasts to root surface. In addition to enhancing fibroblast attachment in a periodontal defect, EMD may directly enhance proliferation of non-mineralizing PDL fibroblasts into the defect, a crucial aspect for enhanced periodontal ligament regeneration. But even more importantly, EMD may initiate the migration of PDL fibroblast to the periodontal defect by enhancing the affinity of the cells for PDGF-BB. The additive effects of EMD and PDGF-BB in increasing wound-fill rates in a dose-dependent manner suggest a possible benefit in combining an extracellular matrix such as EMD and a cytokine growth factor such as PDGF-BB in a board periodontal defect to achieve even greater predictability in the regeneration of a lost periodontium. Further studies combining EMD and PDGF-BB therapy may expend the finding of the latest in vitro studies to the clinic.

Rodrigues et al. (73) evaluated the effects of EMD, TGF- $\beta$ 1, and a combination of both factors (EMD+ TGF- $\beta$ 1) on periodontal ligament fibroblasts, and it has been shown that

EMD induced PDL fibroblast proliferation and migration, total protein synthesis, ALP activity, and mineralization, while TGF- $\beta$ 1 increased cellular adhesion. However, the combination of both factors did not positively alter PDL fibroblast behavior.

Ashkenazi and Shaked (74) evaluated the efficiency of Emdogain® (EMD) in preserving the size of the periodontal ligament progenitor pool (clonogenic capacity) and in promoting their proliferation, and it has been shown that EMD decreased the percentage of PDL fibroblasts with capabilities of arising colonies with 75-100% confluence probably by increasing their differentiation.

Thus, it could be stated that EMD, during several mechanisms, may affect positively the PDL cells in such a way, that the periodontal regeneration might be enhanced. However, the effect of EMD on gingival fibroblasts is a little bit different and may need further studies.

## **THE EFFECT OF EMD ON OTHER CELL TYPES**

The effect of EMD on PDL cells in vitro has been examined in different studies, which found that EMD stimulates cellular proliferation, ALP activity, mineralized nodule formation and TGF- $\beta$ 1 production (21,55,56). However, only a few studies have reported the effect of EMD on other types of cells.

In vitro investigations suggest that EMD may affect the biologic response of periodontal-related cells, including osteoblasts and their precursors, the bone marrow stromal cells (BMSCs), which could play a crucial role in the regenerative process.

To examine the effect of EMD on osteoblast-like cells in vitro, Van den Dolder et al. (75) conducted a study using rat bone marrow cells precultured in osteogenic medium. Then, the cells were harvested and seeded in 24-well plates at a concentration of 20,000 cells/well. The wells were either precoated with 100  $\mu$ g/ml EMD, or left uncoated. The seeded cells were cultured in osteogenic medium for 32 days and analyzed for cell attachment (by using the Live and Dead assay), cell growth (by determining DNA content) and cell differentiation (by measuring alkaline phosphatase activity and calcium content, and by using scanning electron microscopy and the reverse transcription-polymerase chain reaction). According to the result of this study, it has been concluded that EMD had no significant effect on the cell growth and differentiation of rat bone marrow cells.

In a study of Ohyama et al. <sup>(76)</sup>, the effect of EMD on the differentiation of pluripotential mesenchymal cells (C2C12 cells) had been examined. The cells were cultured in 5% serum-containing medium to induce cell differentiation, either with or without the addition of EMD. It has been shown that C2C12 cells (subclone of C2 myoblasts) cultured in differentiation medium without EMD altered their phenotype to myoblasts, exhibiting positive reaction to desmin and myosin heavy chain (the markers of myoblasts). However, the cells cultured in the presence of EMD were strongly inhibited from developing into myoblasts, and showed high ALP activity. The mRNA expression of osteocalcin (a marker of well-differentiated osteoblasts) and type x collagen (a marker of fully differentiated chondrocytes) increased markedly by the EMD-stimulated medium, whereas the expression of desmin, MyoD (transcription factor for skeletal muscle differentiation), and lipoprotein lipase (an early marker of adipogenic differentiation) was drastically decreased.

Takayama et al. <sup>(77)</sup> investigated which molecules in EMD are induced in the differentiation of C2C12 mesenchymal cells. It has been shown that EMD induces both an increased in Cbfa1/Runx2 (a crucial transcriptional factor for osteoblast differentiation) expression and Smad1 (a medium by which Cbfa1/Runx2 and BMP signaling interact) phosphorylation, and that both of these process can be blocked by the BMP antagonist, noggin. Therefore, the ability of EMD to promote osteogenic differentiation may be mediated by BMP-like molecules in EMD, namely BMP2, BMP4 and BMP7.

Keila et al. <sup>(62)</sup> tested the biological effects of EMD on cell number and mineralized tissues formation of rat bone marrow stromal cells. In this study EMD enhanced the osteogenic potential of bone marrow (i.e., stimulated nodule formation and ALP activity), primarily by increasing the number of stromal cells undergoing osteoblastic differentiation. These in vitro effects of EMD are similar to those of prostaglandin E2 (PGE 2), a bone anabolic agent <sup>(78,79)</sup>. It has been suggested that EMD, like PGE 2, maintains the viability of adherent stromal cells and promotes their osteoblastic differentiation.

In another study concerning the effects of EMD on the human bone marrow stromal cells (hBMSCs) in vitro, Song et al. <sup>(80)</sup> found that EMD could promote the proliferation ability of hBMSCs, but has no effect on its attachment and spreading. The same effects of EMD had been found on

porcine bone marrow stromal cells <sup>(81)</sup>. In the same field, Guida et al. <sup>(82)</sup> have shown that EMD significantly increased hBMSCs growth and simultaneously decreased their osteogenic differentiation.

The effect of EMD on the epithelial cell rest of Malassez (ERM) is still not understood. Rincon et al. <sup>(83)</sup> examined the effect of EMD at three concentrations on proliferation, cell attachment and expression of mRNA for two mineralized tissue-related proteins (osteopontin and bone sialoprotein). As for other periodontal cells, the ERM proliferative response was enhanced by EMD. Attachment assays revealed a highly significant increase for ERM and gingival fibroblasts after EMD treatment at all concentration. This study has also shown that EMD stimulated expression of osteopontin mRNA by ERM and alveolar bone cells. The results from this study provide evidence that EMD enhanced cellular event related with proliferation, attachment and osteopontin mRNA expression by periodontal cells, in a manner consistent with its role in periodontal regenerative therapy.

The dental follicle (DF), a loose connective tissue surrounding the un-erupted tooth, is required for eruption to occur <sup>(84,85,86,87,88)</sup>. Beyond their critical role during tooth eruption, follicle cells are also thought to have the ability to differentiate into periodontal cells (cementoblasts, periodontal ligament fibroblasts, and osteoblasts) as required for development of the periodontium <sup>(89)</sup>. A major rationale indicated for using EMD clinically is based on the hypothesis that epithelial-mesenchymal interaction are required for maturation of the developing periodontium and hence for regeneration of periodontal tissues.

The dental follicle surrounding the developing tooth germ is an ectomesenchymal tissue composed of various cell populations derived from the cranial neural crest. Human dental follicle cells (HDFC) are believed to contain precursor cells for cementoblasts, periodontal ligament cells, and osteoblasts. Bone morphogenetic proteins produced by Hertwig's epithelial root sheath or present in enamel matrix derivative EMD seem to be involved in the control of DF cell differentiation, but their precise function remains largely unknown.

Kemoun et al. <sup>(90)</sup> have shown that EMD may activate HDFC toward the cementoblastic phenotype, an effect mainly (but not exclusively) involving both exogenous and endogenous BMP-dependent pathways.

Hakki et al. (91) conducted an in vitro study to further characterize follicle cells and to determine the effect of EMD on these cells. It has been shown that the follicle cells have the capacity to act as cementoblasts or osteoblasts. Furthermore, EMD can regulate follicle cell activity and has been shown to increase osteopontin OPN mRNA and to decrease osteocalcin OCN mRNA expression.

According to the studies mentioned above, it could be stated that EMD enhances the osteogenic potential of bone marrow by increasing the total number of stromal cells, enhances the proliferative response of epithelial cell rest of Malassez and may activate the dental follicle cells toward cementoblastic and/or osteoblastic phenotype.

## **DISCUSSION**

In this review, only in vitro studies were demonstrated, and the results from these studies have been shown to indicate that EMD regulates multiple cell types. EMD enhances proliferation rate, metabolism and protein synthesis, cellular attachment rate, and mineral nodule formation of PDL cells and has a similar influence on cementoblasts and mature osteoblasts. In addition, EMD enhances PDL cell attachment. In contrast to its effects on mesenchymal cells, EMD appears to inhibit the proliferation and growth of epithelial cells. These characteristics partially explain the biological guided tissue regeneration GTR effect attributed to EMD. Most of the effects of EMD are on mature cells rather than on multipotent precursors, suggesting that it may not be capable of controlling the entire periodontal regenerative process.

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