

THE USE OF AN ENAMEL MATRIX PROTEIN DERIVATIVE (EMDOGAIN®) IN REGENERATIVE PERIODONTAL THERAPY. WHICH APPLICATIONS ARE EVIDENCE-BASED? IN-VITRO STUDIES.

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REZUMAT

Scopul terapiei parodontale regenerative este reconstituirea structurilor parodontale pierdute: formarea de cement radicular nou, de ligament parodontal și de os alveolar. Rezultatele din cercetarea fundamentală au arătat rolul important al derivaților proteinelor matricii amelare (EMD) în vindecarea plăgii parodontale. Rezultatele histologice ale studiilor pe subiect animal și uman au arătat că tratamentul cu EMD promovează regenerarea parodontală. Mai mult, studiile clinice au indicat faptul că tratamentul cu EMD influențează pozitiv vindecarea plăgii parodontale la subiecții umani. Scopul trecerii în revistă de față este de a prezenta, bazat pe dovezile existente, indicațiile clinice ale terapiei regenerative cu EMD.

Cuvinte cheie: derivați ai proteinelor matricii amelare, cementogeneză, tratament parodontală regenerativă

ABSTRACT

The goal of regenerative periodontal therapy is the reconstitution of the lost periodontal structures (i.e. the new formation of root cementum, periodontal ligament and alveolar bone). Results from basic research have pointed to the important role of the enamel matrix protein derivative (EMD) in the periodontal wound healing. Histological results from animal and human studies have shown that treatment with EMD promotes periodontal regeneration. Moreover, clinical studies have indicated that treatment with EMD positively influences periodontal wound healing in humans. The goal of the current review is to present, based on the existing evidence, the clinical indications for regenerative therapy with EMD.

Key Words: enamel matrix protein derivative, cementogenesis, regenerative periodontal therapy

INTRODUCTION

Results from basic research have indicated the role of the different types of cementum for attaching the tooth and for the reparative processes in the entire periodontium. Acellular cementum is the most important tissue for the insertion of collagen fibers and plays thereby the largest role in attaching the tooth to the alveolar socket.¹ Studies of Slavkin and Boyde and

Slavkin have shown that proteins, which are secreted during the tooth development by the Hertwig's root sheath, play a crucial role in the formation of acellular root cementum.^{2,3} These proteins referred to as enamel matrix proteins constitute the largest part of the enamel matrix.^{1,4} They consist of a whole family of proteins, from which 90 % are Amelogenin, and the remaining 10% consist of prolin-rich non-Amelogenins, Tuftelin, and other serum proteins.⁴ It has been shown that the chemical structure of Amelogenin remained more or less constant during evolution, even among the individual animal species, exhibiting only slight differences.⁴ Genome sequencing and gene mapping have permitted the identification of HEVIN (SPARC-like 1) as the probable ancestor of the enamel matrix proteins Amelogenin, Ameloblastin and Enamelin.⁵ There are hypothesis that the full-length Amelogenin uniquely regulates proper enamel formation through a process of cooperative mineralization, and not as a pre-formed matrix.⁶ In a series of animal experiments on root development in rats, monkeys and pigs, it

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was immunohistologically demonstrated that the concentration of Amelogenin rises dramatically during tooth development.¹ In addition a close connection between acellular cementum and amelogenin exists.¹ These results have been also confirmed in investigations of human teeth, whereby some histological sections showed a thin layer of highly-mineralized enamel between dentin and root cement. This observation permits the assumption that the attachment of enamel matrix must occur on the dentin surface before the emergence of acellular cementum.¹ Based on these results several *in vivo* experiments in animal models were conducted.¹ In an experiment the lateral incisors of two monkeys were extracted. Immediately after the extraction, a standardized cavity in the root surface was created mesially and distally. The test cavities were then filled with an enamel matrix derivative, while the control cavities remained untreated. All teeth were reimplanted into their original alveoles. Histological evaluation eight weeks after reimplantation resulted in formation of acellular cementum in the defects in which enamel matrix derivative was applied; whereas in the untreated control defects only a reparative, cellular cementum developed.¹ On the basis of these findings the enamel matrix derivative (EMD) from the tooth pouches of not erupted teeth from young pigs were isolated, purified and lyophilized. Since EMD are extreme hydrophobic, they were brought by means of a propylene glycol alginate (PGA) carrier into soluble form before their use in regenerative periodontal therapy.⁷ A recent study has identified enamel matrix proteins and proteolytic enzymes present in EMD and compared them with those extracted from developing porcine enamel itself.⁸ The results have shown that while developing enamel contained amelogenins, albumin, amelin, and enamelin, EMD contained only amelogenins. Thus, at the time being it may be assumed that the main component of EMD are amelogenins.^{4,7,8} A technique or a material must, however, fulfill the following criteria in order to be classified as “regeneration-promoting”:⁹

- *In vitro* studies, which confirm the action mechanism.
- Controlled histological animal studies, which demonstrate formation of new root cementum, periodontal ligament and alveolar bone.
- Human biopsies, which show formation of root cementum, periodontal ligament and alveolar bone on a plaque-infected root surface.
- Controlled clinical studies, which prove a gain of clinical attachment and radiological new bone formation. In the following overview, the existing

evidence regarding the clinical use of EMD is provided.

IN-VITRO STUDIES

Several *in vitro* investigations were carried out to study the mechanism of EMD on the periodontal ligament and gingival fibroblast and on bone cells.¹⁰⁻⁴⁹ Thus, in a series of laboratory studies the migration, attachment, proliferation, biosynthesis activity and formation of mineralized nodules following the application of EMD were examined. Immunoassays were performed too determine the possible presence of existing polypeptide factors.^{7,10} The results have shown that: a) under *in vitro* conditions EMD promotes the proliferation of periodontal ligament (PDL) fibroblasts, but not that of epithelial cells, b) the total protein synthesis of the PDL fibroblasts increases, and c) the formation of mineralized nodules by PDL fibroblasts is promoted. In the mentioned studies no specific molecules such as IGF-1,2; PDGF BB, TNF, TGF β , or IL-1 β could be identified. PDL fibroblasts treated with EMD displayed an increased intracellular cAMP concentration and autocrine releasing of TGF- β 1, IL-6 and PDGF AB in comparison to the control group (without addition of EMD).¹⁴ Although the epithelial cells showed an increased release of cAMP and PDGF AB following the additional application of EMD, their proliferation and growth rate was inhibited.^{12,14} It was concluded that EMD simultaneously promotes the growth of mesenchymal cells by inhibiting that of the epithelial cells and, in the same time, it promotes the release of autocrine growth factors from PDL fibroblasts.¹⁴ Similar findings were also reported by Okubo et al. who demonstrated that EMD has no appreciable effect on osteoblastic differentiation although it stimulates cell growth and IGF-I and TGF- β 1 production in PDL cells.¹⁵ In the MG-63 osteoblast-like cells cultured with EMD, several upregulated and downregulated genes were identified by Carinci et al. the functional activities covered are broad.¹⁶

Palioto et al. have evaluated the effect of EMD, IGF-I, and the combination of these two factors on the proliferation, adhesion, migration, and expression of type I collagen in PDL fibroblasts.¹⁷ The results have indicated that the proliferation of PDL fibroblasts was significantly stimulated by EMD and EMD plus IGF-I in a dose- and time-dependent manner whereas these factors did not affect adhesion, migration, or expression of type I collagen of these cells. Other data indicate that EMD may contain additional mitogenic factors such as TGF- β and BMP-like

growth factors that stimulate fibroblastic proliferation and contribute to the induction of biomineralization during periodontal regeneration.¹⁸⁻²¹ The human periodontal fibroblast response to EMD, amelogenin, platelet-derived growth factor-BB (PDGF-BB) and the combination of these was evaluated by Chong et al.²² In this study, the combination of EMD and PDGF-BB produced greater proliferative and wound-fill effects on PDL cells than each by themselves, but amelogenin did not have significant effects on PDL cell proliferation or migration by itself. The results could suggest that either another enamel matrix component in EMD may be responsible for some of its clinical effects, or that amelogenin alone may not trigger the regenerative potential of the periodontium, requiring the interaction with other enamel matrix components of EMD in guiding the regenerative process.

Keila et al. have investigated the effects of EMD on rat bone marrow stromal cells (BMSC) and on gingival fibroblasts (GF). EMD increased the osteogenic capacity of bone marrow and mineralized nodule formation.²³ The presence of EMD in the initial stages (first 48 hrs) of the culture was crucial for this effect. In contrast, EMD did not induce osteoblastic differentiation of GF but increased up to two-fold both their number and the amount of matrix produced. These results are contradicted by van den Dolder et al, which showed that more BMSC cells were attached to EMD-negative wells, but this effect was no longer apparent at 24 hrs after the beginning of the experiment, both groups showing a similar linear trend of cell growth, with no differences in alkaline phosphatase activity, calcium content, gene expression (osteocalcin, alkaline phosphatase and collagen type I).²⁴ The authors concluded that EMD has no significant effect on the cell growth and differentiation of BMSC. Further investigations have shown that the attaching growth and metabolic rate of PDL fibroblasts increased significantly, when EMD was added in cell cultures and that EMD may convert the differentiation pathway of a pluripotent C2C12 mesenchymal cells into osteoblast and/or chondroblast lineage.^{10-12,14,25} PDL fibroblasts showed a significantly increased alkaline phosphatase activity following the application of EMD and it enhanced human PDL fibroblast proliferation.^{26,27} In the presence of EMD, human PDL fibroblasts showed some morphological changes that made them more similar to cementoblasts than to fibroblasts, suggesting a process of cellular differentiation.²⁷ A recent study examined the influence of EMD on the viability, proliferation, and attachment of periodontal fibroblasts to diseased

root surfaces.²⁸ The results have indicated that cell viability was negatively affected for higher doses over time while low doses displayed viability effects similar to control. PDL cell proliferation appeared to be ameliorated following exposure to EMD and the SEM analysis suggested that cellular attachment to diseased dentin was enhanced following EMD application. Further investigations have demonstrated that EMD significantly increased the mRNA synthesis of the matrix proteins versican, biglycan and decorin and led to an increased hyaluronan synthesis in the gingival and desmodontal fibroblasts.¹¹ It was also suggested that integrins are involved in the interaction of PDL and gingival fibroblasts with EMD.²⁹ However, it has to be emphasized that in most studies EMD had a stronger effect on the desmodontal fibroblasts than on gingival fibroblasts. Other experimental investigations have shown that the application of EMD can regulate the expression of the genes associated with cementoblasts which in turn affects crucially the mineralization process.³⁰ Inoue et al. have evaluated whether the application of EMD to different dental materials which do not normally support cementogenesis such as gutta percha, calcium hydroxide, amalgam, and super EBA cement would alter the in vitro phenotype of PDL cells.³¹ Their findings have indicated that EMD can alter the phenotype of PDL cells when cultured on these materials. Jiang et al. also noted that, while Emdogain-gel stimulates cell proliferation of odontoblasts and osteoblasts, the direct contact between the gel and cells is not required.³² On the other hand, it must be mentioned that some studies have failed to show an influence of EMD upon the proliferation of mouse fibroblasts and marrow stromal cells.³³ Very recent data have also suggested that neither EMD nor PGA has the ability to induce hard tissue and that EMPs contained within EMD might aggregate on the dentin surface and inhibit the effect of the demineralized dentin matrix.³⁴ In a study investigating clot adhesion to protein conditioned root surfaces, human dentin blocks were exposed to either a saturated citric acid solution (CA) or a commercial ethylenediaminetetraacetic acid (EDTA) preparation using standardized protocols.³⁵ Some dentin blocks were additionally conditioned with either bovine serum albumin (BSA) or EMD. Subsequently, fresh human whole blood was applied to the blocks and the blood was allowed to clot before rinsing in phosphate-buffered saline to test adhesion by means of scanning electron microscopy (SEM). The results have indicated that EDTA appeared less efficacious than CA in removing smear layer and in exposing dentin tubules and collagen. Fibrin clot

adhesion was best supported by the CA-treated dentin surface whereas forces produced by the rinse protocol partially removed the fibrin clot from EDTA-treated root surfaces. The results have also indicated that BSA- or EMD treated surfaces poorly retained the fibrin clot and produce a surface morphology similar to that of the smear layer.

Kawase et al examined the effect of EMD on the proliferation of oral epithelium cells (SCC25).³⁶ After 3 days of treatment with EMD, cell division was prevented and at the same time, the cell cycle was stopped in the G1 phase. Additionally, it was shown that the addition of EMD limited significantly the expression of Cytokeratin-18 (CK18). The authors concluded that EMD does not possess a cytostatic but rather, a cytotoxic effect on epithelial cells.³⁶ In an *in vitro* study the combination of 4 mg EMD and active demineralized freeze-dried allogenic bone (DFDBA) showed an increased bone induction.³⁷ It was concluded that EMD possesses no osseoinductive, but rather osteopromotive characteristics when applied in certain concentrations.³⁷ Schwarz et al have shown that EMD stimulates the early stages of the osteoblast maturation by increasing cell proliferation.³⁸ However, when applied on mature cell lines, the main effect was confined to the influence of cell differentiation. A stimulatory role of EMD on mineralized tissue formation by modulating regulatory molecules critical to bone metabolism at the RNA level has also been reported.³⁹

Schwarz et al have investigated the effects of EMD on attachment, proliferation, and viability of human SaOs(2) osteoblasts on titanium implants. The results have indicated that EMD enhanced cell proliferation and viability of human SaOs(2) osteoblasts on SLA titanium implants in a concentration-dependent manner.⁴⁰ Treatment of osteoblasts with EMD significantly stimulated cell proliferation and fibroblast growth factor (FGF)-2 expression but decreased alkaline phosphatase expression.⁴¹ EMD also seems able to create a favorable osteogenic microenvironment by reducing the receptor activator of nuclear factor kappaB ligand (RANKL – the main osteoclast differentiation factor) release and enhancing its osteoblastic decoy receptor - osteoprotegerin (OPG) production.⁴² It was also suggested that EMD may elicit its mitogenic signal through an EMD-specific receptor tyrosine kinase (RTK) towards extracellular signal-regulated kinase (ERK) 1/2.⁴³ In a study that characterized the mitogenic effect of EMD on human gingival fibroblasts and its cooperation with seric growth factors, it was demonstrated that the mitogenic response to EMD

depended on ERK activation.⁴⁴ It seems that EMD treatment may enhance cellular activities of osteoblasts and of osteoclasts which in turn, might support the regeneration of periodontal bony defects.⁴⁵ Itoh et al. specifically studied the mechanisms involved in the enhancement of osteoclasts formation by enamel matrix derivatives.⁴⁶ Their results indicate that EMD induces the formation of osteoclasts through interaction with RANKL, while ERK the p38 mitogen-activated protein kinase (p38 MAPK) may play a critical role in the enhancement of osteoclast formation in a mouse monocytic cell line. Since soluble peptides released from EMD may contribute to the stimulating effects on cell proliferation, a direct contact between EMD and osteoblasts might not be required to induce cell proliferation.⁴⁷ Shimizu et al have examined the ability of EMD to regulate bone sialoprotein (BSP) gene transcription in osteoblast-like cells.⁴⁸ The findings have identified EMD response elements in the rat BSP gene promoter that may mediate the effects of EMD on BSP gene transcription.

A very recent study has evaluated the effect of a combination of a bioactive glass and EMD upon the proliferation and differentiation of the mouse preosteoblastic cell line MC3T3-E1.⁴⁹ Cells were cultured up to 28 days in contact with three types of granules: Bioglass 45S5 granules (BG), 45S5 granules coated with EMD (BG/EMD), and a less reactive glass used as a control (60S). The results have indicated that both BG alone or coated with EMD have the ability to support the growth of osteoblast-like cells *in vitro* and to promote osteoblast differentiation by stimulating the expression of major phenotypic markers. The bioactive granules coated with EMD revealed however, significantly higher protein production than the bioactive granules alone. Pischon et al investigated the effects of EMD on human osteoblasts and PDL cells grown in organoid cultures.⁵⁰ Their results indicate that under organoid culture conditions EMD was able to promote the synthesis of proline-containing proteins such as collagen but no matrix mineralization of primary human osteoblastic cells.

Parkar and Tonetti have evaluated the selective effects of EMD on the activities of 268 cytokine, growth factor, and receptor genes in PDL.⁵¹ The results have indicated that 46% (125 of 268) of the tested genes were found to be expressed by the PDL cells. Of these 125 genes, 38 were differentially expressed by PDL cells which had been cultured in the presence of EMD. Among the 38, 12 were found to be downregulated notably mostly inflammatory genes, whereas 26 genes demonstrated upregulation,

many of these coding for growth factors and growth factor receptors. The results have indicated that EMD down-regulates the expression of genes involved in the early inflammatory phases of wound healing while simultaneously upregulating genes encoding growth and repair-promoting molecules.

It is important to note that certain antibacterial effects and disturbances of bacterial adherence were also found to be influenced by EMD.⁵²⁻⁵⁷ From 24 patients with chronic periodontitis after 4 days of plaque accumulation a plaque sample was taken and divided into 5 equal parts.⁵² Each part was mixed with 5 µl of the following solutions: 1) NaCl, 2) EMD in water, 3) EMD in PGA vehicle, 4) PGA vehicle, 5) Chlorhexidine digluconate (CHX). Subsequently, the vitality of the plaque flora was evaluated under the vital fluorescent microscope. The results have shown that EMD in the PGA vehicle had a very strong antibacterial effect. It was concluded that the antibacterial effect of EMD is mainly due to the effect of the PGA carrier. These findings were later confirmed in an observer-blind, randomized, five-cell crossover study demonstrating for the first time a direct influence of EMD on the vitality of supragingival dental plaque *in vivo*.⁵³ In a further investigation it was shown that EMD inhibits the growth of the periodontal pathogenic bacteria *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*. 24 hours following the application of EMD no living colonies of these pathogenic bacteria could be observed. Moreover, EMD demonstrated no negative effect on gram positive bacteria.⁵⁴ The inhibiting effect of EMD on periodontal pathogenic bacteria was also confirmed by others.^{55,56} Recent data also suggest that *Porphyromonas gingivalis* diminishes the effect of EMD on PDL cells *in vitro* through a cooperative action of gingipains.⁵⁷ Rincon et al have evaluated the influence of EMD on cultured gingival fibroblasts, periodontal ligament fibroblasts and dermal fibroblasts, using an *in vitro* model of wound healing.⁵⁸ The results have demonstrated that cells *in vitro* fill an empty space by a combination of proliferation and cell migration thus indicating that EMD may exert an influence on cells involved in wound healing. In a study in rabbits Mirastschijski et al have primarily investigated the *in vivo* effects of EMD on skin wound healing.⁵⁹ Secondly, they examined the *in vitro* effects of EMD on dermal fibroblasts and on microvascular endothelial cells. Full-thickness, circular 2-cm skin wounds in white 16-week-old rabbits were treated three times weekly with EMD (30mg/ml) in the vehicle propylene glycol alginate or with the vehicle alone. EMD treatment increased the

amount of granulation tissue and accelerated time to complete epithelialization by 3 days compared to the vehicle treatment. In cultured fibroblasts, vascular endothelial growth factor levels in conditioned media were increased more than fivefold with EMD treatment over control, measured by specific enzyme-linked immunosorbent assay. EMD also increased release of matrix metalloproteinase-2 more than threefold from fibroblasts and from endothelial cells. It was concluded that EMD significantly accelerated wound closure in rabbits, possibly by increasing levels of growth factors and proteinases important for granulation tissue formation and granulation. It was shown that EMD may express some angiogenic effects which may play an important role in early wound healing.⁶⁰ Recent results have pointed to the anti-inflammatory properties of EMD which attenuated the release of TNF- α and interleukin-8 in whole blood from healthy donors challenged by lipopolysaccharide or peptidoglycan have been also reported.⁶¹ Furthermore, it was shown that EMD inhibits the attachment of a typical breast cancer cell line (MCF-7) to a bone matrix, thus suggesting that EMD might be useful as an anti-adhesive agent for breast cancer cells to bone *in vivo*.⁶²

The induction and possible subsequent effect of human antibodies against porcine EMD after the first surgery, thereby reducing the clinical effect of secondary surgery, was questioned by Yuan et al in their study, the authors demonstrated that human antibodies against porcine EMD were elicited at ten days after the surgery.⁶³ The results demonstrated that the application of EMD could induce antibodies against different isoforms of porcine amelogenin in humans, but this did not hinder the production of TGF-beta1, which is one of the *in vitro* functions of EMD on PDL fibroblasts.

In conclusion the data from *in vitro* studies strongly indicate that EMD affects important wound healing mechanisms. However, at present, it appears that the underlying molecules and mechanisms are still not completely understood.

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