Review

Emdogain® in carcinogenesis: a systematic review of *in vitro* studies

Matti Laaksonen[1], Timo Sorsa[1] and Tuula Salo[2]

[1]Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Institute of Dentistry, University of Helsinki, Helsinki, Finland
[2]Department of Diagnostics and Oral Medicine, Oulu University Central Hospital, Institute of Dentistry, University of Oulu, Oulu, Finland

Abstract: Emdogain is a commercial product of unknown composition and is clinically used to induce periodontal regeneration. This study aims to review current knowledge of the *in vitro* effects of Emdogain on oral tissues and, in particular, factors related to carcinoma. A systematic approach was used to review studies from the Embase and Pubmed databases; a total of 76 studies were included. These comprised *in vitro* studies of the cytokines in, or regulated by, Emdogain and assays designed to study the effects of EMD on human cells in oral tissues or malignant cells. Several studies have shown that EMD regulates the proliferation, migration, adhesion, gene expression, and cytokine production of (pre-)osteoblasts, periodontal fibroblasts, and gingival fibroblasts. However, the effects of EMD on malignant oral cells are not well understood. EMD seems to have broad regulatory effects on malignant cells and on several carcinoma-related factors. Evidence suggests that patients with premalignant or malignant mucosal lesions should not be treated with EMD. (J Oral Sci 52, 1-11, 2010)

Correspondence to Dr. Tuula Salo, Department of Diagnostics and Oral Medicine, Oulu University Central Hospital, Institute of Dentistry, University of Oulu, P. O. Box 5281, 90014 University of Oulu, FIN-90014 Oulu, Finland
Tel: +358-8-5375592
Fax +358-8-537 5560
E-mail: tuula.salo@oulu.fi

Background

Repair and regeneration of periodontal tissues is a major goal in periodontal treatment. However, periodontal regeneration is a complex, multifactorial process involving molecular interactions in and between mesenchymal and epithelial cell lines, which include periodontal ligament cells, bone cells, gingival fibroblasts, and epithelial cells (1-2). The essential features of successful regeneration are *de novo* cementogenesis, osteogenesis, and assembly of periodontal ligaments onto the acellular cementum. The apical proliferation of epithelial cells must also be simultaneously arrested (3-5). In 1997, a report found evidence that extracted porcine enamel matrix proteins could induce new cementum and bone formation in periodontal defects in monkeys (6). Since then, this tissue extract preparation consisting of hydrophobic enamel matrix proteins from porcine developing enamel has been marketed under the commercial name of Emdogain® (EMD). Numerous *in vivo* and *in vitro* studies, as well as clinical trials, have shown that EMD is clinically useful in promoting periodontal regeneration (7).

Most of these studies have focused on the clinical outcomes of EMD treatments, and the *in vitro* studies have mostly concentrated on periodontal ligament cells or bone cells. However, the effects of EMD on other cell lines, particularly malignant cell lines, have not been adequately investigated. Despite the lack of studies and the fact that EMD is known to have growth factor-like effects on different cell lines, EMD is said to be clinically safe (8-10). However, assessments of clinical safety have been limited to evaluations of the capability of porcine enamel

proteins to induce immunological reactions or local postoperative symptoms, such as pain or sensitivity in the teeth involved, which have been studied both *in vitro* and *in vivo* (8-13). There is abundant evidence regarding the effects of EMD on periodontal tissues, including its stimulation of periodontal and gingival fibroblast cell migration and proliferation. Therefore, EMD might also be capable of inducing alterations in mucosal and malignant tissues. Our rationale for this hypothesis arose from our experience treating a patient in whom a dysplastic oral mucosal lesion progressed to invasive oral carcinoma four months after regenerative EMD treatment. In addition, we have previously shown that EMD enhances the production of MMP-2 and -9 by oral carcinoma cells, and that it is capable of inducing the migration and *in vitro* wound closure of carcinoma cells, along with increased metastasis formation *in vivo* (14). Herein, we review current knowledge on the effects of EMD on oral tissues, particularly with respect to carcinoma.

Systematic Review

Search strategy

An information specialist-assisted search was made of the PubMed and EMBASE databases. The search terms were "Emdogain and/or 'enamel matrix derivative'", and the language was limited to English. The search was restricted to articles published from January 1997 through December 2008. In total, 403 publications were retrieved. No clinical reports concerning associations between Emdogain and oral carcinoma were found. Thus, the review was restricted to *in vitro* studies and the criteria for a true systematic review were applied after the exclusion of *in vivo* studies.

The exclusion criteria were:
1) Article not written in English
2) Clinical study, e.g., studies dealing with radiology or treatment techniques, outcomes, or surveys
3) *in vivo* and *in vitro* studies on histology, immuno-histochemistry, etc.
4) *in vitro* studies involving the study of bacteria or cells other than oral cells or malignant cells
5) Review articles and conference reports.

Altogether, 76 *in vitro* studies were included in this review, which was designed to obtain all available biological data relevant to carcinoma-related factors and the behavior of oral cells. Thus, the inclusion criteria were:
1) Article published in English between 1 January 1997 and 31 December 2008
2) Study of assays designed to investigate human cells present in oral tissues or malignant cells
3) Study of assay designed to investigate cytokines in, or regulated by, Emdogain.

Emdogain and mesenchymal cells
Emdogain and bone-derived cells

As expected, there were many *in vitro* studies of the effects of EMD on bone-derived cells, 37 of which met the inclusion criteria. Alveolar bone cells, including osteoblasts, osteoclasts, and their precursors, play a crucial role in the regenerative process. It is thought that during the development of the periodontium, dental follicle cells have the ability to differentiate into periodontal ligament fibroblasts, cementoblasts, and osteoblasts (15-19). EMD is capable of stimulating follicle cell proliferation and enhancing the expression of bone sialoprotein (BSP) and osteopontin (OP) transcripts (18,20). Furthermore, EMD promotes osteogenic differentiation of pluripotent mesenchymal cell lines into the osteoblast and/or chondroblast lineages and stimulates osteogenesis- and chondrogenesis-related transcription factors (21,22). EMD also enhances bone marrow stromal cell proliferation and osteogenic potential by increasing alkaline phosphatase activity and nodule formation (23,24). However, in contrast to these observations, EMD had no apparent effects on cell growth and differentiation in rat bone marrow cells (25,26). Finally, EMD has been shown to increase the capability of bone marrow stromal cells to differentiate into cementoblasts (27).

The majority of viability studies conclude that EMD stimulates the growth, proliferation, and mobility of osteoblastic cell lines and cementoblasts, and regulates their gene expression (28-38). In addition, EMD plays a role in the differentiation of osteoblasts by regulating the expression of phenotype markers, including BSP, cellular alkaline phosphatase (ALP), and osteocalcin (28,34,36,39-43). However, the findings of these studies are inconsistent, and the effect of EMD osteoblast phenotype markers is unclear. Observations suggest that EMD stimulates proliferation during the early stage of osteoblastic maturation and enhances differentiation in mature cells.

EMD has been found to exert effects on cytokines produced by osteoblasts. It stimulates TGF-β1, connective tissue growth factor (CTGF), and fibroblast growth factor 2 (FGF-2) expression in human osteoblastic cells (39,42,44,45). In addition, both EMD and TGF-β1 protect osteoblasts from inflammation-induced apoptosis (46). Increased COX-2 expression and decreased matrix metalloproteinase (MMP)-1 expression have also been demonstrated (44). Furthermore, EMD has been shown to contain bone morphogenetic protein (BMP)-like growth factor (47-49). EMD has prominent effects on the

endochondral pathway, as it can stimulate proliferation of chondrocytes and chondrogenic differentiation (50-51).

Findings regarding the effects of EMD on bone resorption are somewhat inconsistent. Receptor activator of NF-kB ligand (RANKL) is expressed on the surface of osteoblasts, and the binding of RANKL to the RANK receptor on osteoclast progenitor cells leads to osteoclast differentiation. It has been shown that EMD reduces RANKL release by osteoblasts and enhances production of osteoblastic osteoprotegerin (OPG), thus inhibiting osteoclastogenesis and osteoclast function (32,36). Furthermore, the RANKL mRNA levels of periodontal ligament fibroblast are significantly decreased by EMD (52). However, it has also been shown that EMD induces osteoclast formation via interaction with, and stimulation of, RANKL expressed by osteoblastic cells (53,54). In conclusion, studies suggest that EMD is capable of creating a favorable micro-environment for bone regeneration through its effects on bone formation and resorption. Depending on their maturation stage, EMD influences the differentiation of mesenchymal cells into hard tissue-forming cells and enhances their growth and proliferation.

Emdogain and fibroblasts

A total of 22 studies on the effects of EMD on fibroblasts fulfilled the inclusion criteria. The studies were fairly consistent with regard to the effects of EMD on periodontal ligament (PDL) and gingival fibroblasts: EMD enhances proliferation, migration, and *in vitro* wound healing (23,55-64). EMD can also induce matrix and total protein synthesis by fibroblasts (55,62,65). The mitogenic signaling pathway by which EMD affects PDL fibroblasts has been associated with rapid phosphorylation of the MAP kinase family and nuclear accumulation of smad2, and with activation of EMD-specific receptor tyrosine kinase (RTK) and extracellular signal-regulated kinase (ERK) (63,64,66-68). ERK activation by EMD has been shown to be related to elevated epidermal growth factor receptor expression (EGFR) by fibroblasts (68). Furthermore, EMD stimulates insulin-like growth factor (IGF-I), transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), and interleukin-6 (IL-6) production in PDL fibroblasts (56,59). In addition, EMD induces a greater than threefold increase in MMP-2 levels produced by PDL fibroblasts (69). At the gene level, EMD downregulates genes involved in the early inflammatory phase of wound healing, while simultaneously upregulating growth- and repair-related genes (70). In addition, EMD inhibits caspase activation in gingival fibroblasts, thereby inhibiting TNF-α-induced apoptosis and enhancing survival (71). In angiogenesis, EMD enhances vascular endothelial growth factor (VEGF)

release by PDL fibroblasts (69,72).

The effect of EMD on the differentiation of fibroblasts is more complex. It has been shown that EMD has no effect on ALP activity (59); however, EMD both decreases (57) and increases (62,73,74) ALP activity in PDL fibroblasts. The production of osteopontin and osteoprotegerin has been shown to be increased by EMD (60,74). In addition, although EMD enhances bone-like nodule formation, it does not induce osteoblastic differentiation of PDL or gingival fibroblasts (23,55,59,62). EMD stimulates mineralized tissue formation by modulating regulatory molecules in fibroblasts, a process crucial to bone metabolism (52). Furthermore, EMD alters phenotype markers of PDL fibroblasts when cultured on gutta-percha and calcium hydroxide (75).

Emdogain and epithelial cells

There are a very limited number of studies on the effects of EMD on benign epithelial-derived cells; only 5 such studies were included. It is assumed that during periodontal development, molecules from Hertwig's epithelial root sheath induce differentiation of mesenchymal precursors to form periodontal tissues, and that EMD can mimic this process (16,76). The proliferation of epithelial cell rests of Malassez (ERM) is enhanced by EMD (77). In addition, enhanced osteopontin expression by ERM cells has been observed after EMD treatment (77). However, EMD had no effects on proliferation of rat tongue epithelial cells (55). An assay of *in vivo* wound healing in rabbits revealed significantly enhanced epithelialization after EMD treatment (69). We have found that EMD stimulates the production of MMP-2 and MMP-9 by benign keratinocytes (14).

The results of studies on the effects of EMD on endothelial cell proliferation are somewhat conflicting. One study found that EMD stimulates endothelial cell proliferation (72), while another observed no effects on proliferation (78). Both studies noted that EMD increased chemotaxis of endothelial cells (72,78). EMD also enhanced vascularization around collagen implants in a mouse model (78). In addition, EMD enhanced MMP-2 production in endothelial cells by more than threefold (69).

Emdogain and malignant cells

Only a few experiments have been conducted using EMD and malignant cell lines; 12 were included in this review. Unfortunately, most of these studies investigated cells that are very different from those in oral tissues. Thus, their findings are not of major relevance in the context of EMD and oral cancer. The studies concluded that EMD has either no, or an inhibitory, effect on the

Table 1 *In vitro* effects of Emdogain on carcinogenesis-related cytokines and cellular processes in malignant and benign cell lines

Cytokine or cellular process	Malignant cells	Benign cells	Effect of the cytokine/process on carcinogenesis
MMP-1	+	–	BM and ECM degradation
MMP-2	0, +	+, +, +	BM and ECM degradation, bad prognosis
MMP-3	+		
MMP-8	0		BM and ECM degradation
MMP-9	+	+	BM and ECM degradation, bad prognosis
MMP-13	0		BM and ECM degradation, bad prognosis
MMP-14	0		BM and ECM degradation
Collagenolysis	+		BM and ECM degradation
IL-6	+, +	+	Inflammation regulation, anti-apoptosis
Transformig growth factor-b (TGF-b)	0, +, +	+, +, +, +	Tumorigenesis, immune suppression, bad prognosis
Vascular endothelial growth factor (VEGF)		+, +	Angiogenesis, metastasis, chemoattraction
Platelet-derived growth factor (PDGF)	+	+	Angiogenesis
Basic fibroblast growth factor (bFGF)		+	Angiogenesis, metastasis
Connective tissue growth factor (CTGF)		+	Tumorigenesis, angiogenesis, metastasis
Osteopontin		+, +, +, +, +, +	Tumorigenesis, metastasis, bad prognosis
cAMP	+, –	+	Proliferation, apoptosis
Cell proliferation	0, 0, 0, –, –, –	×	Tumorigenesis, invasion, metastasis
Wound fill	+, +	×	Tumorigenesis, proliferation and migration
Adhesion	0, 0, –	×	Tumorigenesis, invasion, metastasis

The symbol [+] corresponds to a stimulatory effect, [–] to an inhibitory effect, [0] to no change, and [×] to a non-relevant effect of EMD on the corresponding carcinogenesis-related variable. Each symbol ([+], [–], and [0]) corresponds to one study, or to one cell line, when several cell lines were studied.

proliferation of malignant cell lines (Table 1). No differences in the proliferation of fibrosarcoma HT-1080 cells or breast cancer-derived MCF-7 cells were observed after EMD treatment (55,79). EMD decreased proliferation of human cervix-derived epithelial HeLa cells, human osteosarcoma MG-63 cells, and human squamous cell carcinoma-derived SCC-25 cells (39,56,80). One study found that EMD dose-dependently arrested the SCC-25 cell cycle at G1 and did not discernibly increase SCC-25 apoptosis during eight days of treatment (80). Our previous findings on invasive tongue squamous cell carcinoma HSC-3 cells demonstrated that EMD has no effects on carcinoma cell proliferation after 12 h to four days of treatment (14).

Despite the negligible or inhibitory effect of EMD on the proliferation of malignant cell lines, EMD enhances many other cellular functions (Table 1). EMD treatment enhances wound fill rates of MG-63 osteosarcoma cells, *in vitro*, as well as those of invasive tongue squamous cell carcinoma HSC-3 cells (14,81). EMD had no effect on cell adhesion (14,55). However, one study found that EMD inhibited the adhesion of breast cancer (MCF-7) cells to bone matrix (79). The effects of EMD on intracellular cAMP levels differ in epithelial HeLa and SCC-25 cells (56,66). In HeLa cells, the production of PDGF-AB was enhanced by EMD, whereas no significant effect on TGF-β1 was observed (56). In contrast, TGF-β1 levels in culture medium of MG-63 osteosarcoma cells were increased by EMD (39,82). In addition, EMD stimulates phosphorylation of MAP family kinases in SCC-25 cells, as does also TGF-β1 (66,83). Regarding the other cytokines, EMD enhances IL-6 production, but has no effect on the production of BMP-2 or IGF-I by MG-63 cells or HeLa cells (56,82).

We have recently shown that EMD enhances MMP-2 and MMP-9 production by cultured tongue carcinoma cells and stimulates their migration and *in vitro* wound closure (14). Goda et al. (2008) have shown that Emdogain enhances the *in vitro* degradation of type I collagen by malignant MG-63 cells (84). In addition, EMD stimulated the production of MMP-1 and MMP-3, but had no effect on the production of MMP-2, -8, -13, or -14 (84). Furthermore, we found EMD to promote metastasis formation in athymic mice bearing human tongue carcinoma xenografts (14).

Discussion

The purpose of this study was to review the effects and mechanisms of EMD on oral cell lines, carcinoma-related factors, and cytokines. There is a good overall understanding of the effects of EMD on periodontal tissues, but many relevant issues remain unresolved, including the cytokine

content of the product. At the same time, the understanding of the factors contributing to periodontal regeneration and tooth development is not completely clear. Evidence suggests that epithelial-mesenchymal interactions are important during the development and regeneration of periodontal tissues, and that EMD can mimic these processes (74). In carcinogenesis, epithelial-mesenchymal interactions are uncontrolled and one of the first steps towards malignancy is the epithelial-to-mesenchymal transition (EMT) (85). After initial formation of an epithelial malignancy, tumor growth depends on the proteolytic enzymes and cytokines produced by stromal cells surrounding the tumor (85,86). As indicated earlier, EMD stimulates the production of several cytokines that are related not only to periodontal regeneration but also to carcinogenesis (Table 1).

Although, the exact composition of EMD is not known, it has been established that it contains the same proteins that are secreted by Hertwig's epithelial cells, namely, amelogenin and non-amelogenin proteins, including enamelin, tuftelin, and ameloblastin (19,32,39). Amelogenin has growth factor-like effects, which may partially explain the observed effects of EMD (87). However, EMD has also been found to be more effective than amelogenin in enhancing fibroblast proliferation, together with other cellular functions (88,89). Therefore, it is generally assumed that EMD contains bioactive factors other than the enamel proteins (48,65,88). Several studies have attempted to identity the cytokines in EMD, with varying results. Kawase et al. (65,80), and later Suzuki et al. (48), identified TGF-β1, or TGF-β-like substances, in EMD, which they believed to be the main functional components. Furthermore, EMD enhances the production of TGF-β1 in many different cell lines, including osteoblasts, fibroblasts, and malignant osteosarcoma cells (39). TGF-β1 has broad regulatory functions, but it has also become clear that TGF-β1 can act as one of the principal cytokines in carcinogenesis (90,91). During tumor progression, TGF-β1 and other cytokines (those in the epidermal growth factor (EGF) families, for example) enhance the morphological and invasive phases of the EMT phenotype (92). Furthermore, TGF-β1 promotes MMP-mediated oral cancer invasion and activates several MMPs (92-94).

Along with TGF-β1, MMPs are crucial in tumorigenesis (95). Typically, several MMPs are expressed in human malignant tumors, and elevated MMP levels are correlated with tumor aggressiveness and invasiveness and with poor prognosis (96). MMPs play an important role in all stages of tumorigenesis: they enhance tumor-induced angiogenesis, regulate and activate growth factors, and break down extracellular matrix and basement membrane to allow tumor cell invasion and metastatic spread (96,97). We have recently shown that EMD is capable of enhancing MMP-2 and -9 release by HSC-3 oral carcinoma cells (14). In addition, EMD enhances both the production of MMP-1 in malignant MG-63 osteosarcoma cells and the *in vitro* degradation of type I collagen (84). In benign cells, EMD can also enhance the production of MMP-2 by endothelial cells and fibroblasts (69). This effect of EMD on MMPs is of great interest and indicates that EMD may have carcinogenic properties.

EMD also contains a small number of other cytokines. These include a BMP-like growth factor, which belongs to the TGF-β family, and BSP-like molecules (47,48,82). Furthermore, the production of several growth factors is enhanced by EMD. These include CTGF and FGF-2 produced by osteoblasts; IGF-I, PDGF, VEGF, and IL-6 produced by PDL fibroblasts; and PDGF and VEGF produced by epithelial-derived cells. In addition, EMD enhances the production of several cytokines, including ALP, osteoprotegerin, osteopontin, and bone sialoprotein in mesenchymal cell lines and osteopontin in epithelial cells (18,77). Most of these factors are associated with carcinoma growth and metastasis. IGF-I is believed to regulate carcinoma cell proliferation and to influence tumor growth (98,99). BMPs have been associated with high-risk premalignant and malignant lesions of oral epithelium, with salivary gland tumors, and with the promotion of tumor cell migration (100,101). Bone sialoprotein and osteopontin are highly upregulated in oral squamous cell carcinoma and have been linked to different stages of tumor progression, including metastasis. Furthermore, bone sialoprotein and osteopontin can specifically bind and activate MMP-2, MMP-3, and MMP-9 (102,103).

Neoangiogenesis, or the growth of capillary vessels, is crucial for the spread of carcinoma (97). A significant increase in vascularity can be seen during the transition from normal oral mucosa to dysplasia to invasive carcinoma. EMD can affect angiogenesis by enhancing the release of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) by PDL fibroblasts and epithelial cells (56,69,72). Furthermore, EMD directly enhances the chemotaxis and angiogenesis of endothelial cells *in vivo*. VEGF induces endothelial proliferation, migration, and specialization in new and developing vascular beds (104). It is also a very potent promoter of neoangiogenesis in many types of tumor (105). PDGF has VEGF-like effects on angiogenesis. Therefore, several new tyrosine kinase inhibitors targeting the VEGF pathway, as well as PDGF, are currently in advanced clinical development (106).

As mentioned above, we recently demonstrated that EMD enhances migration and MMP-2 and MMP-9 production in HSC-3 carcinoma cells, and that it promotes tumor metastasis *in vivo* (14). The hypothesis of that study arose from our clinical experience treating a patient with chronic periodontal disease and dysplastic mucosal lesions. We used EMD to treat a residual periodontal pocket, which led to clinical worsening and spreading of the dysplastic lesions in the EMD-treated region. Carcinoma was diagnosed at the same site four months after the regenerative therapy. As we have stated, there were predisposing factors: the patient already had widespread dysplastic premalignant non-homogeneous leukoplakia lesions, which are capable of rapid progression to carcinoma. However, the unusual location of the initial cancer within bone – close to the apex of d.12 – and the temporal and spatial proximity to the EMD-treated site provoked our suspicion. Based on the results of this review, the possibility of a link between EMD treatment and the induction of cancer in this patient cannot be ruled out.

A limitation of the current study was that, excluding our previous study, there were very few studies concerning EMD and malignant cells, and none focused on carcinogenesis. Instead, malignant cells were used mainly as an "easy growing" substitute for their benign precursors. However, the existing studies on epithelial and mesenchymal cells lend support to the idea that EMD is capable of enhancing carcinoma-related factors, in particular cytokine production. Thus, further studies of the effects of EMD on oral carcinoma cells, along with studies focusing on the composition of EMD, are needed before the safety of EMD can be confirmed. It should be remembered that detectable amounts of EMD remain at the site of application on the root surface for at least two weeks, and that local application enables the use of high dosages (107). Furthermore, a systematic Cochrane review suggests that the overall treatment effect of EMD might be overestimated and that the clinical advantages of using EMD are unproven (108).

In conclusion, EMD was found to enhance the *in vitro* production of cytokines related to carcinogenesis in malignant and benign cell lines. In addition, EMD stimulates other cellular functions and processes in malignant cells, but may partially inhibit proliferation. Because there is limited knowledge of the effects of EMD on carcinoma tissues, and the exact cytokine content of the product is unknown, we believe that the use of EMD is contraindicated in patients with oral carcinoma or mucosal dysplasia.

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