Enamel matrix proteins exhibit growth factor activity: A review of evidence at the cellular and molecular levels

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Abstract. Enamel matrix derivative (EMD) is a commercially available protein extract, mainly comprising amelogenins. A number of other polypeptides have been identified in EMD, mostly growth factors, which promote cementogenesis and osteogenesis during the regeneration processes through the regulation of cell proliferation, differentiation and activity; however, not all of their functions are clear. Enamel extracts have been proposed to have numerous activities such as bone morphogenetic protein- and transforming growth factor β (TGF-β)-like activity, and activities similar to those of insulin-like growth factor, fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor and epidermal growth factor. These activities have been observed at the molecular and cellular levels and in numerous animal models. Furthermore, it has been suggested that EMD contains an unidentified biologically active factor that acts in combination with TGF-β1, and several studies have reported functional similarities between growth factors and TGF-β in cellular processes. The effects of enamel extracts on the cell cycle and biology are summarized and discussed in this review.

Contents

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1. Introduction

In this review, the growth factor and growth factor-like activities of enamel matrix proteins (EMPs) were examined. Enamel matrix derivative (EMD), a mixture of EMPs, emerged almost two decades ago as an agent capable of periodontal regeneration. Although numerous studies and review papers have been published on this topic, the understanding of the cellular and molecular mechanisms of action of EMD is far from exhaustive (1-7); thus this subject is revisited in the present comprehensive review.

Growth factors regulate important cellular events involved in numerous physiological and pathological processes by binding to specific cell surface receptors (8). A number of polypeptide growth factors have been identified that regulate cell proliferation, chemotaxis or differentiation. Certain growth and differentiation factors, such as insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP)-2, are able to stimulate the cellular activities associated with periodontal regeneration in periodontal ligament (PDL) cells (9-19).

Ameloblasts synthesize and secrete a number of EMPs, including amelogenins, ameloblastin, amelotin, tuftelin and enamelin (20,21). EMPs are associated with the process of amelogenesis and they play a crucial role in the formation of enamel and periodontal attachment during tooth development; however, in wound healing and tissue regeneration EMPs show several novel functions.

Clinically, EMPs are applied as an extract of porcine fetal tooth enamel matrix (known as EMD) for the periodontal regeneration of teeth affected by periodontitis, root coverage procedures and tooth implantation. EMD has also been used in *in vitro* research, for dentin repair, tooth movement, anti-cancer treatment evaluation and skin wound healing (22). The regeneration of several types of periodontal tissue, including alveolar bone, cellular cementum and collagenous ligaments, and the formation of an extracellular matrix (ECM) layer in adjacent tissues has been observed following EMD application (22). The predominant (>90%) component of EMD is amelogenin, which in its native form is slowly degraded by the proteinases enamelysin and kallikrein-4 (20). Cleavage produces different, shorter forms of amelogenin. These amelogenin-derived peptides are classified into two groups: Leucine-rich amelogenin peptide (LRAP) and tyrosine-rich amelogenin peptide (TRAP) (Fig. 1). While EMD presents a number of growth factor-like effects, it is the TGF-β activity that is predominately known (23-26). EMD has also been reported to contain other cytokines, such as a BMP-like growth factor and bone sialoprotein (BSP)-like molecules (27,28).

To date, which of the EMD fractions are crucial for BMP- or TGF-β-like activity has not been definitively clarified. In one study, the chromatographic separation of EMD resulted in 22 protein fractions. Fractions 4-6 had BMP-like activity while fractions 8-13 had TGF-β-like activity, and fractions 4-13 were found to contain 10-25-kDa peptides (26). It was observed that BMP-2 signal transduction activity was inhibited by authentic TGF-β1 and the TGF-β1 or TGF-β-like activity in an EMD gel, but that signal transduction by TGF-β was not suppressed by BMP-2. It was hypothesized that TGF-β could not completely inhibit the activity of BMP, since BMP and TGF-β activate SMAD intracellular transcription factors (26). In oral epithelial cells and fibroblasts isolated from gingiva, EMD stimulated the rapid translocation of SMAD2 protein from the cytoplasm to the cell nucleus, which suggests the involvement of TGF-β-like factors (24).

2. TGF-β in the periodontium

TGF-β is a member of the TGF-β superfamily, which consists of five isoforms of TGF-β and associated homologous proteins including activins, inhibins, BMPs, growth differentiation factors and the glial cell line-derived neurotrophic factor family (29-31). These structurally related polypeptides are characterized by the presence of a common sequence and specifically positioned structures, namely a 'cystine knot' composed of six cysteine residues (32). The structural differences between the TGF-β proteins and the BMPs have been found to lie within four regions of the polypeptide chain. These are the N-terminal segment, the loops at the end of fingers 1 and 2 and the C-terminus of helix α3 (33). In addition, the receptor activation differs, despite the fact that in each case it involves the recruitment of pairs of type I and type II receptor molecules by dimeric ligands to form signaling complexes. Sequential binding is typical of TGF-β and activin ligand receptors, while a fully cooperative interaction is characteristic of BMP ligand receptors (33). Mature TGF-β is a homodimer, composed of two 12.5-kDa polypeptides joined by a disulfide bond between two cysteine 77 residues and by hydrophobic interactions (34).

TGF-β regulates various cellular processes including cell growth, apoptosis, homeostasis, differentiation, migration, wound healing, fibrosis, angiogenesis and carcinogenesis (35-37). TGF-β1 has been indicated to play an important role in the modulation of tissue formation and development of the periodontium (38). It is also a transcription-regulating factor (29,30,39,40). Notably, the response can differ considerably according to the type of cell and the stimulation context, even though the activation is induced by the same receptor. It is, therefore, critical, particularly in carcinogenesis, to know where TGF-β may act as a suppressor and where as a stimulator. The role of TGF-β in carcinogenesis appears to involve a signaling pathway involving SMAD proteins, which is induced by TGF-β (41). Non-SMAD signaling pathways in TGF-β signaling include extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), Rho-associated protein kinases and P21-activated kinase-2, depending on the cell line (42). It has been suggested that TGF-β-mediated apoptosis is regulated by the modulation of SMAD activation (43). Furthermore, TGF-β has been indicated to participate in carcinogenesis by immune suppression (44). Several studies have indicated that TGF-β arrests growth in the majority of cell types (29,45). This effect has been observed in primary embryonic fibroblasts; however, in fibroblasts from SMAD3-null mice, the growth inhibitory effect of TGF-β was suppressed (45).

Several studies of EMD have demonstrated that it contains TGF-β1 or a TGF-β-like substance, and that EMD rapidly translocates SMAD2, an effector of the TGF-β signaling pathway, into the nucleus and modulates the proliferation of human gingival fibroblasts and oral epithelial cells in a cell type-specific manner (24,26,46,47). Furthermore, experiments *in vitro* on epithelial and fibroblastic cells with anti-TGF-β antibodies, in which the TGF-β1-induced SMAD2 translocation was blocked, showed that the EMD-induced translocation of SMAD2 was strongly reduced. This may indicate that they act via the same mechanism (48). In human PDL fibroblasts, EMD stimulated the release of TGF-β1 (45). PDL cell metabolism was significantly increased when EMD was present in cultures, and an increased autocrine production of TGF-β1, interleukin 6 (IL-6) and PDGF-AB was detected when compared with that in control cultures (49).

It has been postulated that EMD may contain an additional mitogenic factor, which acts in combination with TGF-β1 to fully stimulate fibroblastic proliferation. Kawase *et al* (46) investigated the effects of EMD, TGF-β1 and neutralizing TGF-β antibody on epithelial and fibroblastic cells. It was found that porcine EMD translocated SMAD2 into the nucleus of cells, as does TGF-β1 or a TGF-β-like substance. SMAD2 is an effector of the TGF-β signaling pathway that modulates the proliferation of gingival fibroblastic and oral epithelial cells. In the study, cells were treated with porcine TGF-β1 in order to compare its actions with those of EMD. In the epithelial and fibroblastic cells, TGF-β1 replicated the action of EMD in the nuclear accumulation of SMAD2, the phosphorylation of mitogen activated protein (MAP) kinase family members and, consequently, cell growth induction. Neutralizing TGF-β antibody blocked certain actions of EMD. The anti-TGF-β antibody prevented TGF-β1-induced SMAD2 translocation and blocked other actions of EMD, such as p38-MAP kinase

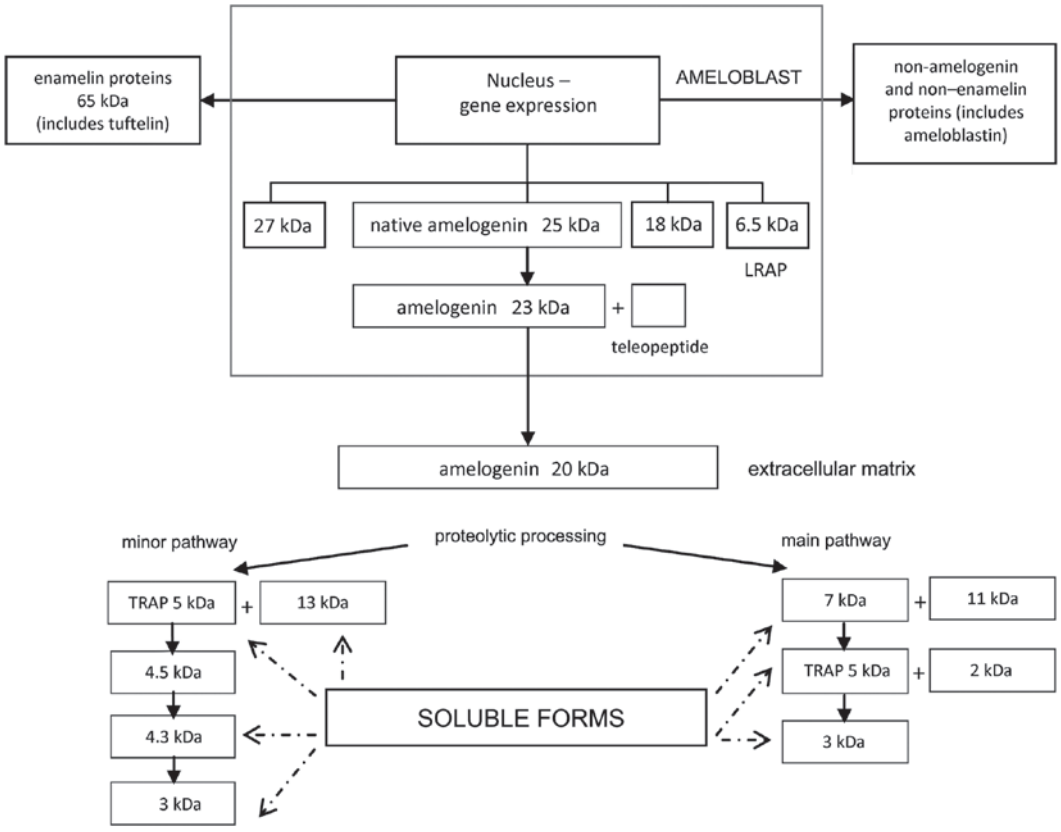

Figure 1. Extracellular amelogenin proteolytic process. Ameloblasts synthesize three categories of enamel matrix protein: Amelogenin, enamelin and a non-amelogenin non-enamelin group. Amelogenin is slowly degraded by specific extracellular proteolytic enzymes to smaller soluble forms. LRAP, leucine-rich amelogenin peptide; TRAP, tyrosine-rich amelogenin peptide.

phosphorylation and p21WAF1/cip1 expression in epithelial cells. It has been suggested that TGF-β1 or a TGF-β-like substance is a principal bioactive factor in EMD, but the TGF-β1-neutralizing antibody did not block EMD-induced fibroblast proliferation, strongly implying that EMD contains unidentified mitogenic factor(s).

The effects of EMD vary according to the origin of the cell line; EMD has been found to increase the proliferation of gingival fibroblasts but decrease the proliferation of epithelial cells (50,51). Notably, no apoptotic effect was observed when epithelial cells were treated with EMD (51), which led to the conclusion that EMD acted as a cytostatic rather than a cytotoxic agent for epithelial cells. EMD has also been demonstrated to have a growth-inhibitory effect on epithelial (HeLa) cells and human squamous cell carcinoma-derived-25 cells (51). Kawase *et al* (46) postulated that EMD reduced DNA synthesis, suggesting that a reduction in epithelial cell growth could be mediated by TGF-β-like activity. EMD and TGF-β are also able to stimulate the production of matrix metalloproteinases (MMPs), which are crucial in tumorigenesis and in benign keratinocytes (27).

The findings concerning the effect of EMD on the other special epithelial cells, endothelial cells, which are required for the healing and regeneration of periodontal tissue, are contradictory and include either a stimulatory effect (52) or no effect at all (53) on proliferation. A low concentration of EMD stimulated the proliferation and migration of endothelial cells, whereas a higher concentration inhibited them (54). It was hypothesized that TGF-β present in the EMD-conditioned media may be responsible for the effects of EMD on the proliferation and viability of human umbilical vein endothelial cells (54).

The effect of EMD is also dependent upon its specific fraction. Full-length amelogenin molecules have been shown to stimulate the autocrine production of BMP, while smaller fractions like LRAP and TRAP stimulate the autocrine production of TGF-β in the human PDL (4). The TGF-β protein, however, has not been found in the composition of EMD (50). The aforementioned studies suggest that specific amelogenin molecules may stimulate the autocrine release of growth factors that coordinate the regenerative effects of EMD.

3. BMP in the periodontium

BMPs belong to the TGF-β superfamily of growth factors (55) but have two extra domains in addition to the structure typical of the TGF family members TGF-β, activin/inhibin and nodal. The family of BMPs is particularly noteworthy due to its function in the morphogenesis and development of various tissues, cell proliferation, apoptosis and ECM protein synthesis, as well as its ability to induce cartilage and bone formation (56-59). BMP-2, -4, -6 and -7 have osteoinductive activities *in vivo* (60) whereas other BMPs exhibit low osteoinductive activity but modulate BMP action (57). BMP-2 and -4 are also naturally expressed in the skin, in epidermal keratinocytes and dermal fibroblasts (61,62). In addition, they induce embryonic stem cells

into chondrogenic differentiation (63). The function of BMP-2 extends to affecting the production of ECM, since it stimulates the production of glycosaminoglycan and collagen type II (64). It acts through influencing the balance between MMP2 and its inhibitor, tissue inhibitor of metalloproteinase-2 (65), as well as via the classical SMAD pathway through which it can co-interact with FGF and VEGF (66-68).

BMPs bind to BMP receptors of types I and II. Type I receptors include activin receptor-like kinase (ALK)-2, ALK-3 (BMP receptor IA; expressed in most types of cells) and ALK-6 (BMP receptor IB; chondrocytes and osteoclasts express only this type of BMP receptor), and mainly determine the specificity of the intracellular signals. Type II receptors include BMP type II receptor, activin type II receptor and activin type IIB receptor. BMPs activate intracellular transcription factors SMAD-1, -5 and -8, which dimerize with SMAD-4 prior to translocation into the nucleus (67,69-71). Osteopontin, osteoprotegerin (OPG), BMP-7 and SMAD-1 are activated by BMP through the SMAD activation mechanism (72-74). BMPs also stimulate MAP kinase, phosphoinositide-3 kinase and JNK by SMAD-independent signals (75,76).

In the periodontium the presence of BMP-2 and BMP-4 between sections of human periodontal structures is distinct. Immunohistochemistry has shown intense staining in the PDL with almost no detection in the cementum, alveolar bone and gingival connective tissue (77). This finding did not correlate with the expression of mRNA for these proteins. *In vitro* the gingival and PDL fibroblasts expressed mRNA for BMP-2 and -4, and while the BMP-4 mRNA level was similar in the gingival and periodontal fibroblasts, the BMP-2 expression was higher in the gingival fibroblasts (77).

EMD has been shown to contain or stimulate growth factors such as TGF-β, BMP-2, -4 and -7 (24,26,78-80). It has also been noted that amelogenin stimulates BSP gene transcription in osteoblasts by inducing the expression of nuclear proteins that bind to FGF-2 response elements and TGF-β1 activation elements in BSP gene promoters (4). Amelogenin has comparable osteogenic activities to recombinant human BMP-2 and induces the formation of a reparative dentin bridge, in a manner comparable with that of BMP-7 and calcium hydroxide (81). In response to EMD treatment, human dental follicle cells have exhibited increased expression levels of BMP-2, BMP-7, BSP and two cementum markers, namely cementum attachment protein and cementum protein-23 (78).

The investigation of osteoprogenitor cells (C2C12) and human microvascular endothelial cells showed that noggin, a molecule that prevents BMPs from binding to their receptors (82,83), abolishes alkaline phosphatase activity in C2C12 cells. This suggests that the effect on osteoprogenitor cell differentiation results from the action of BMP-like proteins, whereas the effects on proliferation and angiogenesis are associated with lower molecular weight proteins from EMD (84). By contrast, the osteoinductive activity of LRAP has been found to be comparable with the effect of BMP-2 on the osteogenesis of mouse embryonic stem cells (85).

4. VEGF in the periodontium

VEGF induces endothelial proliferation, migration and specialization in new and developing vascular beds (86) during embryogenesis and later development, wound healing and menstruation. It is also a potent promoter of angiogenesis in numerous types of tumors (87), diabetes, rheumatoid fever and psoriasis (88).

In the periodontium, VEGF has been shown to be involved in the regulation of bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation (89). VEGF has been found in a higher concentration in crevicular fluid during gingivitis (90). Angiogenesis is central to tissue healing. EMD, directly or indirectly, positively influences this process. EMD has been shown to have a chemotactic effect on endothelial cells *in vitro* (53) and to stimulate human microvascular endothelial cells (HMVECs) as well as their production of VEGF (52,84). Additionally, EMD enhances the communication between HMVECs and PDL fibroblasts (52). Human periodontal and dermal fibroblasts cultured with EMD also exhibit increased VEGF production (52,91). One of the possible mechanisms by which EMD stimulates angiogenesis is by increasing the expression of MMP-2 in human microvascular pericytes (91,92). Another route could be through the EMD-induced stimulation of VEGF production, which occurs partially via TGF-β1 and FGF-2 in human gingival fibroblasts (37). It is notable that EMD and its major component, amelogenin, stimulate angiogenesis but the small tyrosine-rich and leucine-rich polypeptides present in a 5-kD protein fraction derived from EMD do not (93). This stimulation is dose-dependent (94). At low concentrations EMD stimulates PDL fibroblast proliferation by HMVECs but in higher concentrations it does not (52).

5. PDGF in the periodontium

PDGF stimulates the activation of proliferation, migration and matrix synthesis in gingival and PDL fibroblasts, cementoblasts, pre-osteoblasts and osteoblasts in a dose- and time-dependent manner (15,95-100). It is suggested that during wound healing, PDGF cooperates with other growth factors, such as IGF-1 (101), TGF-β (99) or VEGF (102). PDGF has VEGF-like effects on angiogenesis. The three main VEGF receptors are structurally similar to the family of PDGF receptor III class of tyrosine kinase receptors (RTK class III) (103). The RTK-ERK 1/2 signaling pathway induced by EMD is similar to that activated by epidermal growth factor (EGF) (103). PDGF upregulates the expression of integrin collagen receptors in rat fibroblasts (104) and also stimulates actin filament reorganization in cytoskeletal proteins (105). EMD induces PDL fibroblasts to secrete TGF-β1, IL-6 and PDGF-AB by intracellular cyclic adenosine monophosphate signaling; epithelial cell growth is inhibited by the same signal (49). *A* combination of EMD and PDGF-BB produced greater proliferative and wound-fill effects on PDL cells than either protein by itself (106).

6. FGF in the periodontium

FGF acts as a mitogen for vascularization during organogenesis (107,108). FGF is specifically upregulated in bone marrow stromal stem cell transplants, and may play important roles in the growth of blood vessels and in the recruitment of hematopoietic elements (109,110). Additionally, the actions of bFGF

and VEGF are complementary and when present together may result in a synergistic effect on angiogenesis (111-113), which may involve enhanced cytoprotection against complement-mediated vascular injury (114). It has been indicated that FGF-2 promotes valve interstitial cell wound repair through the TGF-β/SMAD-2 and -3 signaling pathway (115). EMD has been observed to increase the expression of FGF-2 and TGF-β1 in osteoblasts (116). FGF-2 stimulates the proliferation of osteoblastic cells but reduces their differentiation; therefore, EMD may modify cell proliferation and differentiation via FGF-2 production (117,118). EMD potentially induces FGF-2 via prostaglandin-2 production, decreases the expression of MMP-1 via FGF-2 (119) and modulates FGF-2 gene expression in osteoblasts and Müller cells (120,121). In smooth muscle cells, FGF-2 induces the expression of MMP-1 protein and inhibits collagen synthesis (122). The cooperative effects of FGF-2 and VEGF have also been explored in periodontal ligament cells (123).

7. EGF in the periodontium

EGF enhances the cellular proliferation and differentiation of epidermal and epithelial cells, fibroblasts, cartilage and bone-derived cells during growth, maturation and healing processes (124-129), and is also a potent mitogenic factor (130-132). The treatment of human gingival fibroblasts with EMD results in an autocrine/paracrine EGF receptor (EGFR) transactivation. There are two suggested independent mechanisms of EGFR transactivation: i) An intracellular pathway mediated by the src family of non-receptor tyrosine kinases (133,134); and ii) an extracellular pathway mediated by the shedding of a transmembrane pro-form of EGFR ligands by metalloproteinases (135-137). The capacity of PDL cells to bind to EGF and EMD has been assessed in a ^{125}I-EGF radioligand binding assay. The assay showed that there was no significant competitive binding between EGF and EMD, indicating that the EGFR is not the binding site for EMD (103). These results indicate that EMD does not contain biologically effective amounts of EGF and supports a study in which no EGF was detected in EMD by radioimmunoassay (50). Other studies have demonstrated cross-talk between TGF-β and EGF-stimulated pathways (138) and suggested that the molecular mechanisms by which TGF-β1 and EGF interact to elicit these phenotypic changes may involve MAP kinases, SMADs, activator protein and upstream stimulatory factor transcription factors (139,140).

8. IGF-1 in the periodontium

IGF-1 is a multifunctional peptide that regulates growth, differentiation and the expression of ECM proteins (9). It is also thought to be a key mediator of wound healing, inducing epithelial and mesenchymal cell proliferation (8). IGF is reported to stimulate cell migration (126,130,132) and has been successfully used for dentine-pulp complex regeneration (141). EMD stimulates IGF-1, TGF-β1, PDGF and IL-6 production in PDL fibroblasts (49,142); however, it has no effect on IGF-1, BMP-2 or IL-6 in HeLa and MG-63 cell lines (49,143). PDGF and IGF together synergistically enhance gingival fibroblast contractility, and may have had a synergistic effect on wound healing (101,144). Furthermore, cementum-derived growth factor (CGF) has been characterized as an IGF-1-like molecule (145). CGF has been shown to be mitogenic for both PDL and gingival fibroblasts, to promote the migration and growth of progenitor cells adjacent to the dentin matrix, and to participate in their differentiation into cementoblasts (146).

9. Conclusion

The effects of EMD on periodontal tissue regeneration have been well documented, however, the mechanism of action remains unknown. To date, no receptors specific for amelogenin have been identified, to the best of our knowledge. However, there are putative receptors, such as lysosomal-associated membrane proteins (LAMP); LAMP-1 interacts with LRAP, and LAMP-3 with longer amelogenin protein isoforms. Notably, neither of these receptors interacts with both of the amelogenin molecules (147). The role of amelogenin derivatives in the periodontium is also unclear. Studies carried out on LRAP have shown its induction effect on the expression of bone acidic glycoprotein-75, BSP (148) and OPG in mineralized tissues, including cementoblasts (149). When evaluating whether the action of EMD on cells is dependent on direct cell-matrix contact or mediated by growth factors released from EMD or stimulated by it, the close interaction between growth factors presents a challenge. It has been suggested that the soluble growth factors contained in EMD may be responsible for the stimulating effects. TGF-β and small amelogenin peptides are potential candidates for the factors mediating the action of EMD (150), however further studies are required to investigate this further.

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