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The influence of enamel matrix derivative on the angiogenic activity of primary endothelial cells

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Background and Objective: Angiogenesis plays a crucial role in early wound healing and tissue regeneration. Although enamel matrix derivative (EMD) has demonstrated the potential to stimulate periodontal regeneration, the biological effects of EMD on angiogenesis and underlying mechanisms have not been fully elucidated. The aim of the present study was to examine the angiogenic effects of EMD *in vitro*.

Material and Methods: Human umbilical vein endothelial cells (HUVECs) were used to assess the effect of EMD on proliferation, survival, adhesion and migration. The effect of EMD on HUVEC angiogenesis was assessed by a three-dimensional sprouting assay. In order to understand the signalling mechanism of altered cell proliferation of HUVECs caused by EMD, the phosphorylation status of ERK1/2 and of the serine/threonine protein kinase Akt was analysed by western blot using phospho-specific antibodies.

Results: The proliferation of HUVECs was stimulated by 50 μg/mL EMD, whereas higher concentrations (≥ 100 μg/mL) resulted in an increased apoptotic rate. The mitogenic response to EMD was associated with the activation of ERK1/2. Enamel matrix derivative did not affect cell adhesion, but all concentrations of EMD tested (0.1–250 μg/mL) promoted migration of HUVECs. Furthermore, EMD induced capillary-like sprout formation from HUVEC spheroids in a dose-dependent manner.

Conclusion: Our data indicate that EMD acts as a proangiogenic factor *in vitro* and, as such, might contribute to periodontal tissue regeneration by stimulation of vessel formation during wound healing.

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A major goal of periodontal treatment is to control periodontal infection and to eliminate remaining anatomical deformities by regenerating the affected periodontal structures. By definition, periodontal regeneration involves the reconstruction of a functionally oriented periodontal ligament inserting into new alveolar bone and cementum over a previously diseased root surface (1). Various procedures have been considered for periodontal regeneration, including root surface modification, the use of bone grafts or bone substitutes, guided tissue regeneration, combined approaches and biological mediators (2–7). However, significant heterogeneity in outcome variables was observed across studies, demonstrating that a complete and predictable regeneration of periodontal tissues is still difficult to obtain (8,9). At the cellular level, the process of

periodontal tissue regeneration requires selective migration (chemotaxis), proliferation and differentiation of various cell types, particularly osteoblasts, cementoblasts, gingival fibroblasts and periodontal ligament cells, epithelial cells and endothelial cells. This process is complicated by the fact that periodontal wound healing occurs on the nonvascular and nonvital hard tissue of the root surface. Thus, periodontal tissue regeneration critically relies on the re-establishment and proper function of the damaged vascular system. This process, referred to as angiogenesis, is initiated by the sprouting of endothelial cells from pre-existing blood vessels and requires degradation of the extracellular matrix, migration, proliferation and organization in capillary networks (10). Angiogenesis is stimulated by local factors in the microenvironment of wounds, including low pH, reduced oxygen tension and increased lactate levels (11,12). The subsequent growth of new blood vessels into the wound site allows the delivery of oxygen, nutrients and essential growth factors to the injured tissues and formation of granulation tissue (13). Thus, angiogenesis is crucial to accelerate wound healing and regeneration of lost tissues in periodontal therapy.

A biomimetic approach to promote periodontal regeneration has utilized enamel matrix derivative (EMD) that has been purified from porcine enamel matrix protein extracts and is composed mostly of amelogenins and related proteins (14). Recent data from a systematic Cochrane review provide evidence that EMD is effective in enhancing clinical outcomes following treatment of human intrabony defects (15). In addition, findings from histological studies demonstrated the ability of EMD to promote periodontal regeneration (16,17). Bosshard (18) suggested in a recent review that EMD affects many different cell types and has effects on cell proliferation and survival, attachment, spreading and chemotaxis, as well as the expression of transcription factors, growth factors, cytokines and extracellular matrix molecules. Furthermore, EMD affects the expression of molecules involved in the regulation of bone remodelling. However, only a few studies investigated the biological impact of EMD on vessel formation and angiogenesis. In particular, EMD was found to stimulate the outgrowth of new blood vessels and to stimulate proliferation, chemotaxis and gene expression of vascular cells (19–21). In contrast, Yuan *et al.* (22) observed no proliferative effect of EMD on endothelial cells but only a dose-dependent chemotactic effect. Likewise, Bertl *et al.* (23) found that EMD exerted no stimulating effect on human umbilical vein endothelial cell (HUVEC) proliferation and had a concentration-dependent effect on the viability of HUVECs. Thus, the biological effects of EMD on angiogenesis and its underlying molecular mechanism in cultured human endothelial cells remain unknown.

The purpose of the present study was to explore the angiogenic influence of EMD on primary endothelial cells using HUVECs as a cellular model; therefore, we studied various angiogenic parameters, such as cell proliferation, apoptosis, adhesion, chemotaxis and spheroid sprout formation, in order to suggest a molecular explanation for the effects of EMD on primary endothelial cells and to determine its role in angiogenesis during wound healing.

Material and methods

Preparation of EMD

The Emdogain® (EMD) was supplied by Straumann (Basel, Switzerland). The EMD was suspended in the corresponding medium without serum and further diluted at varying concentrations.

Cell culture

Pooled HUVECs were purchased from Lonza (Wuppertal, Germany) and cultured in endothelial basal medium (Lonza) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor, gentamicin sulfate, amphotericin-B and 10% fetal bovine serum (PAA, Pasching, Austria) until the third passage. Cells were then detached by trypsinization, seeded at a density of 300,000 cells per 60 mm dish and grown at 37°C in a 5% CO_2 atmosphere at 100% humidity for 48–72 h until confluency.

Bromodeoxyuridine (BrdU) proliferation assay

The HUVEC proliferation rate was determined by BrdU incorporation over night using a cell proliferation ELISA (Roche, Basel, Switzerland) according to the manufacturer's guidelines. In brief, HUVECs were plated on 35 mm Petri dishes coated with phosphate-buffered saline (PBS), various concentrations of EMD (0.1–250 μg/mL) and fibroblast growth factor (FGF; 100 ng/mL) at 300,000 cells per cell culture dish in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. The culture plates were incubated for 24 h with BrdU at 37°C in a 5% CO_2 atmosphere. Next day, cells were harvested, and BrdU incorporation was measured using the BrdU ELISA. Each experiment was performed in triplicate.

Measurement of apoptosis

In order to detect HUVEC apoptosis after *in vitro* incubation, the cytoplasmic histone-associated DNA fragments were measured using the cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. This assay is based on the quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones. The absorbance of the samples was read with an ELISA plate reader (Tecan, Crailsheim, Germany) at 405 nm wavelength. The results were interpreted as absorbance per gram of protein.

Cell adhesion assay

In order to evaluate the potential of EMD to serve as synthetic extracellular matrices, cell attachment assays using HUVECs were performed. Coated dishes were prepared by covering suspension dishes (Greiner, Frickenhausen,

Germany) with 10 mg/mL bovine serum albumin (BSA; Sigma, St Louis, MO, USA), EMD, fibronectin, collagen, collagen + EMD or fibronectin + EMD in PBS at 4°C overnight. The extracellular matrix proteins fibronectin and collagen served as positive controls, as these proteins are strong mediators of cellular adhesion. Prior to use, plates were washed extensively with PBS, blocked with 1% BSA in PBS for 2 h at 4°C, and were washed again with PBS. The HUVECs (300,000) were seeded on coated or uncoated dishes and incubated at 37°C for 1 h. Unbound cells were removed by washing with PBS three times. Attached cells were stained with 0.2% crystal violet (Sigma) in 10% ethanol, lysed and quantified by determination of absorbance in a microplate reader (Tecan M200, Munich, Germany) at 562 nm. The assays were performed in triplicate and are presented as the average of three data points as plotted in arbitrary units.

Cell migration assay

The effects of EMD on HUVEC migration were measured in a modified Boyden chamber by using a FluoroBlok™ 24-multiwell insert system (BD, Heidelberg, Germany). The inserts of the chamber consist of a light-opaque polyethylene membrane with 8.0 μm pores blocking the transmission of light within the range of 490–700 nm. A total of 300,000 cells per chamber were loaded in 500 μL Dulbecco's modified Eagle's medium. The outer well was loaded with 750 μL Dulbecco's modified Eagle's medium containing various concentrations of EMD (0.1–250 μg/mL), 100 μg/mL BSA or alternatively, 100 ng/mL bibasic platelet-derived growth factor (PDGF-BB; Peprotech, Hamburg, Germany). Cells were allowed to migrate for 24 h in standard conditions at 37°C. Subsequently, cells were fixed in 4% paraformaldehyde (PFA) in PBS and stained with 0.5 μg/mL 4′,6-Diamidin-2-phenylindol (Sigma). The number of cells that had migrated through the FluoroBlok inserts were counted under a fluorescence microscope (Zeiss, Jena, Germany) and plotted as an average of three test samples.

Immunoblotting

For detection of proteins in western blots, monoclonal mouse anti-phospho-Erk (E-4) and anti-actin (C-2) as well as polyclonal rabbit anti-Erk2 (C-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). In addition, rabbit monoclonal anti-Akt and anti-pS-Akt (Ser473) antibodies (Cell Signaling Technology, Boston, MA, USA) were used.

Cells were incubated in the presence of PBS, EMD at concentrations of 0.1–250 μg/mL or FGF (100 ng/mL) in standard culture medium. Protein samples were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) in combination with precast NuPAGE 4–12% or 10% Bis–Tris gels (1 mm) (NuPAGE 4–12% or 10% Bis–Tris gels; Invitrogen) at 200 V according to the manufacturer's guidelines. Following electrophoresis, proteins were blotted to a polyvinylidenfluoride membrane and incubated for at least 1 h in blocking buffer (5% BSA and 1% Tween-20 in Tris-buffered saline). Membranes were incubated over night with appropriate dilutions of primary antibody in blocking buffer. The next day, membranes were washed and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody solution in blocking buffer (dilutions: anti-mouse antibody 1:3000 and anti-rabbit antibody 1:5000; Sigma). After additional washing steps, antibody complexes were visualized on film by Immun-Star AP substrate (Bio-Rad, Munich, Germany).

Spheroid-based angiogenesis assay

Endothelial cell spheroids were generated as previously described (24). In brief, confluent monolayers of HUVECs were trypsinized, and cells were resuspended in endothelial basal medium containing 20% methocel. Subsequently, drops of 30 μL, containing 400 HUVECs each, were seeded on nonadhesive culture dishes and cultured upside down at 37°C (5% CO_2, 100% humidity) for 24 h. In these conditions, endothelial cells formed spheroids, which were harvested and embedded into a collagen-methocel matrix. The EMD was added into matrix (internal application) or applied on top of the matrix (external application). After 24 h, the assay was stopped by addition of 1 mL 4% PFA. Angiogenesis *in vitro* was quantified by measuring the cumulative length of the sprouts that had grown out of each spheroid using an inverse microscope (Olympus IX70) and digital imaging software (NIS elements AR 3.0, Melville, NY, USA).

Statistical analysis

Student's unpaired or paired *t*-test (graph pad PRISM 5.0 software, La Jolla, CA, USA) was used to determine statistical significance. The significance level was set at $p < 0.05$ (*) and $p < 0.01$ (**).

Results

Effects of EMD on proliferation and viability of HUVECs

The effect of EMD on the proliferation of HUVECs was determined by incorporation of BrdU into *de novo* synthesized DNA of proliferating cells and subsequent detection of BrdU by an ELISA. Quantitative analysis demonstrated that the proliferation rate of HUVECs significantly increased at all concentrations of EMD tested in comparison to the negative control, PBS (Fig. 1). Cell proliferation increased upon addition of EMD in comparison to the negative control, PBS, and reached a maximum at 50 μg/mL but decreased with further increasing amounts of EMD. The highest proliferation rates were observed upon treatment of HUVECs with the positive control, FGF, a growth factor known to stimulate proliferation of endothelial cells (25).

In order to unravel the reason for the decrease of HUVEC proliferation upon addition of 100 and 250 μg/mL EMD, its effect on apoptosis of HUVECs was measured using the cell

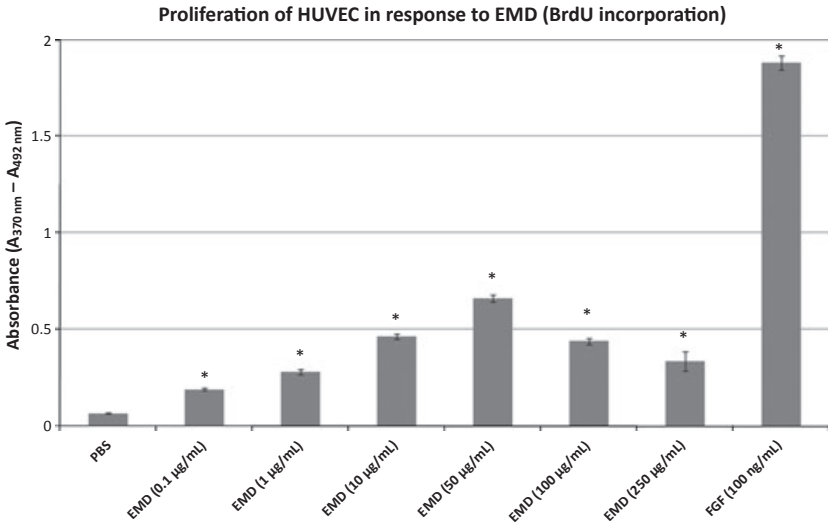

Fig. 1. Effect of enamel matrix derivative (EMD) on proliferation of human umbilical vein endothelial cells (HUVECs). Cells were seeded at 300,000 cells per well and incubated with different concentrations of EMD (0.1–250 µg/mL), a negative control (phosphate-buffered saline; PBS) and a positive control (fibroblast growth factor; FGF; 100 ng/mL). Cell proliferation was assessed by a bromodeoxyuridine incorporation assay after 24 h. The maximal increase in proliferation rate was observed with an EMD concentration of 50 µg/mL. Values are expressed as means ± SEM from three individual experiments. $*p < 0.05$ vs. control.

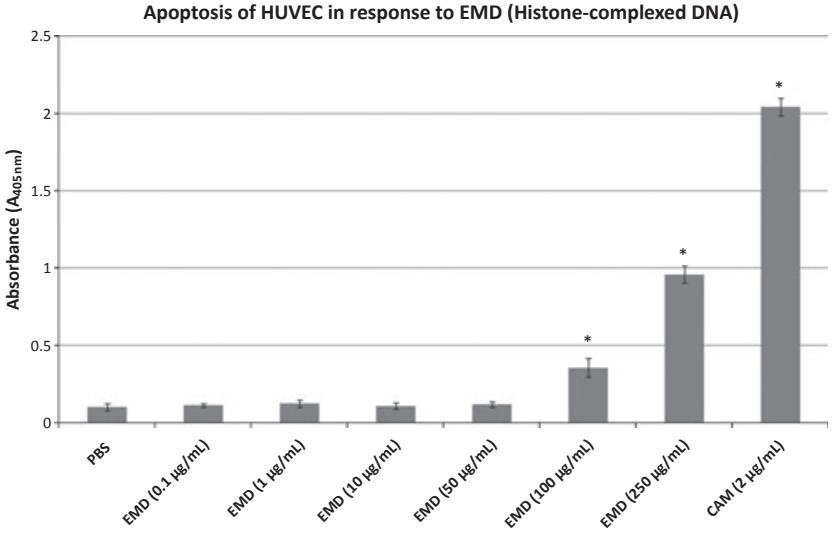

Fig. 2. Effect of EMD on apoptosis of HUVECs. Subsequent to treatment of HUVECs with various concentrations of EMD (0.1–250 µg/mL), the extent of cellular apoptosis was measured using an ELISA-based assay detecting the presence of cytoplasmatic histone-associated DNA fragments. Treatment with camptothecin (CAM; 2 µg/mL) served as a positive control. At high concentrations (≥ 100 µg/mL), EMD induced apoptosis in HUVECs. Data are expressed as means ± SEM from three individual experiments. $*p < 0.05$ vs. control.

death detection ELISA kit (Fig. 2). Camptothecin (2 µg/mL) was used as a positive control to induce apoptosis. A significant increase in apoptotic cells in comparison to the negative control was observed at EMD concentrations > 100 µg/mL. At lower EMD concentrations (0.1–50 µg/mL), no significant induction of apoptosis was detected. These results suggest that EMD concentrations of 100–250 µg/mL decreased proliferation of HUVECs due to the significant induction of apoptosis; therefore, succeeding studies were performed with a maximum of 50 µg/mL EMD.

Effects of EMD on HUVEC adhesion

Enamel matrix derivative was tested for its ability to influence the attachment of HUVECs using an *in vitro* adhesion assay. For this purpose, HUVECs were plated on BSA (a nonadhesive substrate), EMD, collagen, fibronectin, collagen mixed with EMD or fibronectin mixed with EMD. The EMD did not promote adhesion of HUVECs and was in this respect comparable to the negative control, BSA (Fig. 3). However, attachment of HUVECs to plates coated with collagen or fibronectin, which was significantly enhanced, was slightly inhibited by the addition of EMD. In conclusion, EMD did not exert proadhesive effects on HUVECs but altered their adhesion properties to *bona fide* extracellular matrix proteins, such as collagen or fibronectin.

Effects of EMD on migration of HUVECs

The influence of EMD on cell motility was investigated using a modified Boyden migration chamber (Fig. 4). All of the tested EMD concentrations (0.1–250 µg/mL) stimulated cell migration in comparison to negative control (BSA). The EMD induced migration of HUVECs in a dose-dependent manner. At concentrations of 100–250 µg/mL, EMD stimulated HUVEC migration to an extent comparable to the positive control, PDGF-BB. Thus, EMD significantly enhanced HUVEC migration *in vitro* (Fig. 4).

Effects of EMD on activation of ERK and Akt in HUVECs

Cell proliferation usually implies the activation of intracellular signalling pathways, including MAPK or the phosphatidylinositol 3-kinase/Akt

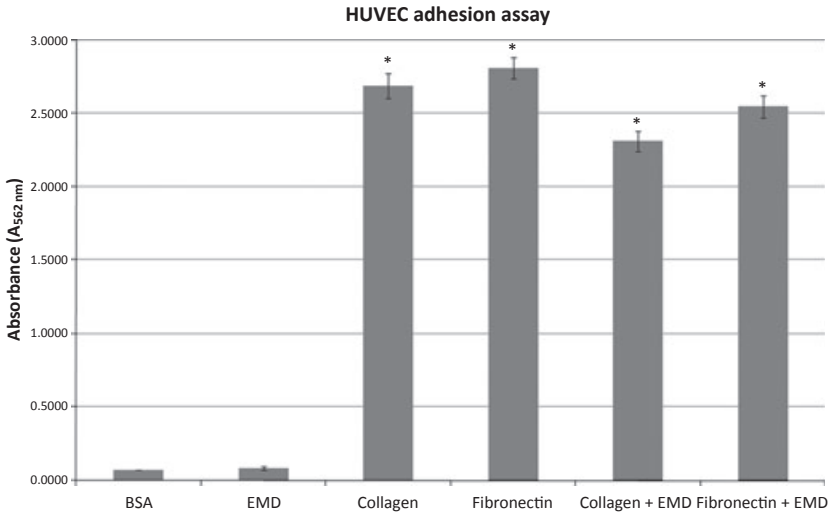

Fig. 3. Effect of EMD on HUVEC adhesion. A total of 300,000 HUVECs were added to wells coated with bovine serum albumin (BSA), EMD, collagen, fibronectin, collagen + EMD or fibronectin + EMD for 1 h at 37°C. Subsequently, unbound cells were washed away and the remaining cells were stained with 0.2% crystal violet. The cells were then lysed, and the solubilized crystal violet incorporated by adherent cells was measured on a plate reader at 562 nm. The EMD did not mediate a significant effect on HUVEC adhesion but decreased adhesion to collagen and fibronectin. Data are presented as means ± SEM from three individual experiments. $*p < 0.05$ vs. control.

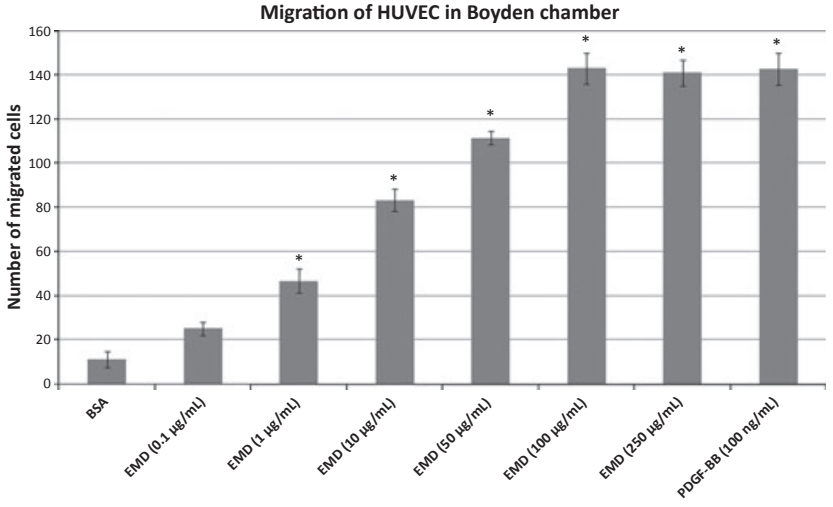

Fig. 4. Effect of EMD on HUVEC migration. The migration of HUVECs was assessed using a modified Boyden chamber. A total of 300,000 cells were allowed to migrate at 37°C for 24 h towards a gradient of EMD (0.1–250 µg/mL), bibasic platelet-derived growth factor (PDGF-BB; 100 ng/mL) as a positive control or BSA (100 µg/mL) as a negative control, which were loaded in the lower chamber. Data obtained yielded a dose-dependent stimulation of HUVEC migration towards EMD and PDGF-BB but not BSA. Data are presented as means ± SEM from three individual experiments. $*p < 0.05$ vs. control.

pathway (26). In order to define whether or not these pathways were involved in the pro-proliferative effects mediated by EMD on HUVECs, we detected the change in the amount of phosphorylated form of ERK and Akt using phospho-specific antibodies. Western blot analyses revealed that the treatment of HUVECs with EMD incrased the phosphorylation of ERK

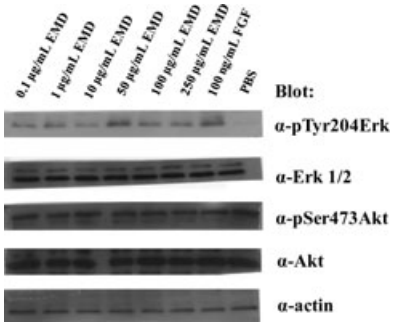

Fig. 5. Influence of different concentrations of EMD on ERK1/2 and Akt phosphorylation in HUVECs. The HUVECs were serum starved for 1 h and subsequently stimulated with EMD (0.1–250 µg/mL) or FGF (100 ng/mL). Cell lysates were subjected to western blot using antibodies specifically recognizing phospho-ERK1/2 (Tyr 204), ERK1/2, phospho-Akt (Ser 473) and Akt. Actin served as an internal control to ensure equal loading of protein in each lane. Representative western blots are presented, showing that EMD induced phosphorylation of ERK1/2 in a dose-dependent manner; however, phosphorylation of Akt was not affected by EMD. Results shown are representative of three experiments.

in a dose-dependent manner (Fig. 5). In contrast, EMD had no effect on Akt phosphorylation or Akt activity in HUVECs. These results suggest that EMD promoted the proliferation of HUVECs, probably by the activation of the ERK pathway, whereas the Akt pathway was not involved.

Effects of EMD on angiogenic sprouting

In order to assess the biological activity of EMD towards endothelial cells, we tested its ability to stimulate angiogenesis *in vitro* using a three-dimensional assay of endothelial cell cultivation. So-called HUVEC spheroids were stimulated to sprout in a collagen gel in the absence or presence of different amounts of EMD, and capillary sprouts grew radially in all three dimensions 24 h after stimulation. In the absence of EMD stimulation, only a few short capillary sprouts originated from HUVEC spheroids (Fig. 6A). The addition of 0.5 µg/mL EMD on the top of the collagen matrix (external application) potently

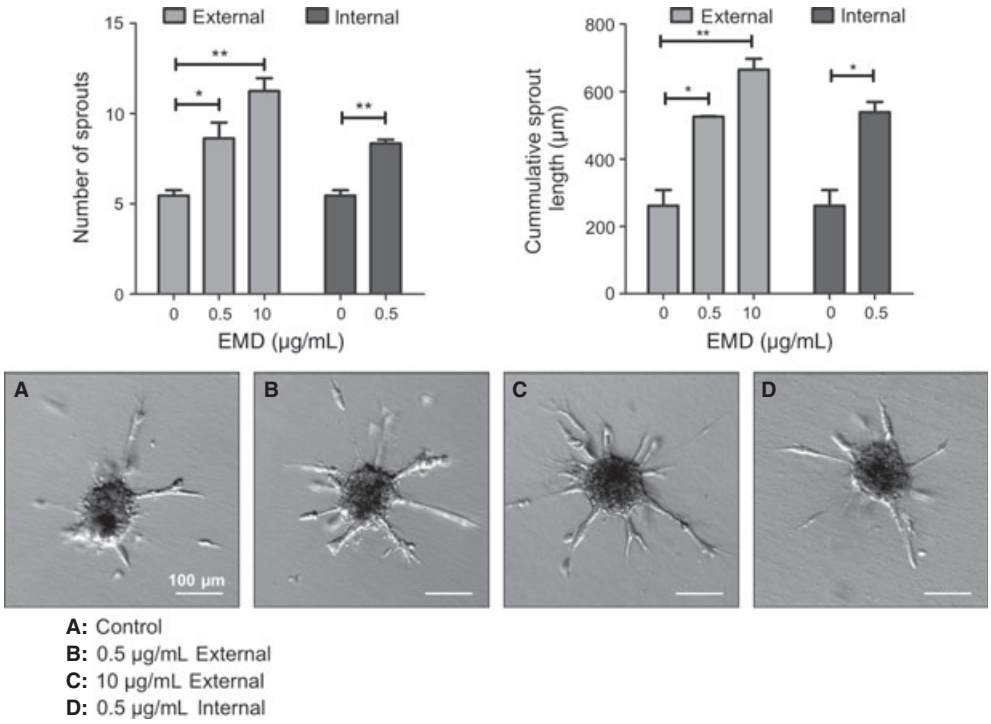

Fig. 6. Effect of EMD on angiogenic sprout formation *in vitro*. HUVEC spheroids were seeded in collagen gels and PBS (control; A), 0.5 µg/mL (B) or 10 µg/mL (C) EMD were applied on top of the matrix (external application). Alternatively, the collagen matrix was loaded with PBS or 0.5 µg/mL EMD (D; internal application). Treatment of HUVEC spheroids with EMD increased the average number of sprouts, as well as the cumulative sprout length. Data are shown as means ± SEM from three individual experiments. *$p < 0.05$ and **$p < 0.01$.

stimulated capillary sprouting and resulted in a significant increase of length and number of capillary sprouts ($p < 0.05$; Fig. 6B). This effect was further potentiated by an increase in EMD up to 10 µg/mL (Fig. 6C). Furthermore, the sprout length nearly doubled upon stimulation with 0.5 µg/mL EMD in comparison to the negative control. Similar effects were observed when EMD was mixed into the collagen matrix at a concentration of 0.5 µg/mL ($p < 0.05$; Fig. 6D). In sum, data indicate that EMD exerts a proangiogenic effect on capillary sprout formation of HUVECs.

Discussion

The healing of damaged periodontal tissues is a complex and integrated sequence of events and requires a high level of co-ordination of various specialized cell types. Thus, the process of wound healing is characterized by a series of overlapping stages that include inflammation, granulation tissue formation and tissue remodelling (13). One of the key elements in granulation tissue formation is angiogenesis or neovascularization (13). Angiogenesis is a biological mechanism of new capillary formation and requires activation, migration and proliferation of endothelial cells from pre-existing vessels (10). In the present study, we investigated the specific effects of EMD on primary endothelial cells and the process of angiogenesis. The HUVEC culture is a well-established *in vitro* cell model for molecular imaging of angiogenesis (27). Data demonstrated that EMD stimulates proliferation and migration of HUVECs but also induces apoptosis at high concentrations. Furthermore, no direct effect of EMD on HUVEC adhesion was observed, but rather EMD modulated adhesion of HUVECs to extracellular matrix proteins. In addition, EMD increased the process of angiogenesis *in vitro* and together, these findings suggest a proangiogenic potential of EMD onto primary endothelial cells *in vitro*.

With respect to the effects of EMD on endothelial cell proliferation, previous studies have yielded controversial results (19–23). While Yuan *et al*. (22) observed no stimulating effect of EMD on proliferation of HUVECs, other studies observed the greatest increase in EMD-stimulated proliferation of endothelial cells at 0.1 (23), 25 (20), < 50 (19) or 12.5–250 µg/mL (21). In the present study, the peak of stimulation occurred at 50 µg/mL, whereas higher concentrations of EMD reduced the overall magnitude of increase below the peak value. The decreased proliferation of HUVECs under exposure to high doses of EMD can be explained by the fact that EMD stimulates HUVEC apoptosis in a dose-dependent manner. Thus, in the present study treatment with 100–250 µg/mL EMD significantly increased HUVEC apoptosis. This implies that EMD exerts cytotoxic effects on HUVECs at higher concentrations. The observation that higher concentrations of EMD have an inhibitory effect on angiogenesis is in accordance with previous data (28). Differences between the studies may

also be related to different donors and different endothelial cell lines, but also due to differences in the preparation and concentration of EMD. Thus, several studies used EMD as a lyophilized powder, whereas in the present study the commercial preparation, which contains EMD and a propylene glycol alginate carrier, was used. Indeed, it has been demonstrated that antimicrobial effects of EMD can be attributed to the vehicle, propylene glycol alginate, and not to the amelogenin fraction (29,30).

Cell adhesion data showed that dishes coated with EMD did not mediate significant attachment of HUVECs. Thus, EMD does not associate with extracellular matrix to support attachment of HUVECs. However, the combination of EMD with fibronectin or collagen resulted in a decrease in cell attachment of HUVECs if compared with single application of these well-known attachment proteins. This is interesting insofar as our succeeding studies implied a proangiogenic potential of EMD, and increased motility of endothelial cells is a prerequisite for angiogenesis (31). Maybe EMD supports vessel growth by reducing cell attachment to *bona fide* extracellular matrix proteins and by increasing motility of primary endothelial cells. Comparable inhibitory effects of EMD on cell adhesion have been observed upon seeding human periodontal ligament cells onto EMD-coated culture dishes (32,33).

The effects of EMD on HUVEC migration described in the present study are consistent with previous studies reporting a dose-dependent increase in HUVEC migration in response to EMD concentrations ranging between 12.5 and 250 μg/mL (21,22). In contrast, Bertl *et al.* (23) found that HUVEC migration was stimulated by EMD concentrations of ≤ 50 μg/mL using an *in vitro* wound healing assay. Schlueter *et al.* (19) reported an increase in human microvascular endothelial cell migration at 100 μg/mL EMD using a microporous membrane system. The differences between the studies may be related to the fact that different migration assays, endothelial cells, culture conditions and EMD concentrations were employed.

In this report, EMD was shown to induce ERK1/2 phosphorylation in HUVECs. This observation is supported by previous data demonstrating that the ERK signalling pathway is a key player in cell proliferation and therefore affects central cellular processes, such as angiogenesis or migration (34). Thus, ERK1/2 phosphorylation was shown to be an integral part of survival in endothelial cells and is regarded to be a proangiogenic event (35). In contrast, stimulation of HUVECs did not result in phosphorylation of Akt at the Ser473 residue, suggesting that this survival pathway is not activated by EMD. Taken together, the positive effects of EMD on HUVECs occur at least in part by stimulation of ERK signalling.

Data obtained from our three-dimensional *in vitro* angiogenesis assay suggested that EMD significantly increased the sprouting capacity of HUVEC spheroids, thus implying a proangiogenic function of EMD. The effects of EMD did not depend on the method of EMD application, because HUVEC sprouting was stimulated by EMD upon mixing into the collagen matrix (internal application) or upon application on top of the matrigel preparation (external application) to allow EMD to diffuse into the matrix. These findings are consistent with several reports in the literature. Using a different *in vitro* angiogenesis assay, Yuan *et al.* (22) detected the stimulation of blood vessel outgrowth in the EMD groups but not in the negative control group. Moreover, immunohistochemical analysis of collagen membranes soaked with EMD and implanted subcutaneously in mice showed significantly more blood vessels compared with the control mice. Schlueter *et al.* (19) also found EMD-induced vessel formation *in vitro* and suggested that EMD promotes angiogenesis directly by stimulation of endothelial cells and indirectly by upregulation of angiogenic factors, such as vascular endothelial growth factor, in periodontal ligament fibroblasts. Furthermore, a study by Johnson *et al.* (20) confirmed the enhancing effect of EMD on angiogenesis *in vitro* using human microvascular endothelial cells. Our findings are further corroborated by recent studies *in vivo* reporting the ability of EMD to induce the formation of new blood vessels. Thoma *et al.* (36) evaluated the angiogenic response of EMD and different protein pools derived from the parent EMD in mice. The greatest angiogenic potential of the EMD parent was observed at a weight of 125 ng, which resulted in a 4.3-fold increase in angiogenesis compared with the negative control. Furthermore, a recent study demonstrated the ability of EMD to induce the formation of new blood vessels in the chorioallantoic membrane of developing chicken eggs, suggesting that amelogenin may be the active component of EMD with respect to the stimulation of angiogenic activity (28). Likewise, the angiogenic effect of amelogenins was evaluated in an *ex vivo* chick aortic arch assay, showing an increased microvessel outgrowth by 76% (37).

The data presented in this study indicate that EMD has a positive influence on angiogenesis, which is a central process in tissue healing. Previous studies attributed the positive influence of EMD on wound healing to their predominant protein, amelogenin (for a review, see Lyngstadaas *et al.*; 38). Using the relaxed dermal equivalent *in vitro* model for early wound contraction, Grayson *et al.* (39) demonstrated that amelogenins augment fibroblast-driven collagen matrix remodelling and increase dermal contraction and fibroblast proliferation. This stimulatory effect of amelogenins on cutaneous wound healing is supported by further studies. In particular, Mirastschijski *et al.* (40) demonstrated accelerated wound closure of full-thickness skin wounds in rabbits following topical application of EMD. This beneficial effect of EMD was already evident in the early wound contraction phase. Moreover, an amelogenin formulation for skin wounds was demonstrated to be beneficial in the treatment of hard-to-heal venous leg ulcers (41). The amelogenins used as an adjunctive treatment of hard-to-heal venous leg ulcers resulted in

significant reductions in ulcer size, reduced wound-associated pain and reduced levels of exudate. Thus, EMD seems to reduce postoperative pain/swelling and to accelerate wound healing, suggesting an anti-inflammatory role of EMD in the process of wound healing. Indeed, more recent work by Myhre *et al*. (42) found that EMD limits the release of proinflammtory cytokines induced by lipopolysaccharide or peptidoglycan in human blood, indicating that EMD has anti-inflammatory properties. Therefore, the beneficial effects of EMD on wound healing may be enhanced by downregulation of inflammatory mediators.

Taken together, the present results provide evidence *in vitro* that EMD exerts proangiogenic effects on human endothelial cells. This proangiogenic effect is based on increased proliferation and mobility of primary endothelial cells and, as such, may contribute to the beneficial effects of EMD on wound healing in periodontal surgery.

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