J Clin Periodontol 2011; 38: 253–260 doi: 10.1111/j.1600-051X.2010.01656.x

Journal of Clinical Periodontology

Angiogenic activity of an enamel matrix derivative (EMD) and EMD-derived proteins: an experimental study in mice

Daniel S. Thoma[1], Cristina C. Villar[2], David L. Carnes[2], Michel Dard[3], Yong-Hee Patricia Chun[2] and David L. Cochran[2]

[1]Clinic for Fixed and Removable Prosthodontics and Dental Material Science, University of Zurich, Zurich, Switzerland; [2]Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA; [3]Institut Straumann AG, Basel, Switzerland

Thoma DS, Villar CC, Carnes DL, Dard M, Chun Y-H, Cochran DL. Angiogenic activity of enamel matrix derivative (EMD) and EMD-derived proteins: an experimental study in mice. J Clin Periodontol 2011; 38: 253–260. doi: 10.1111/j.1600-051X.2010.01656.x.

Abstract

Objectives: To determine whether all or only certain proteins in an enamel matrix derivative (EMD) are angiogenic.

Materials and Methods: The angiogenic effect was analysed using an in vivo angiogenesis assay. Silicon tubes were filled with or without potential and known angiogenic-modulating factors: (i) an EMD parent, (ii) nine pools of EMD proteins, (iii) fibroblast growth factor/vascular endothelial growth factor and (iv) amelogenin. Silicon tubes were implanted subcutaneously in mice. Dextran–fluorescein isothiocyanate (FITC) was injected via the tail vein, mice were euthanized and tubes were retrieved. Neovascularization was determined by measuring the amount of dextran–FITC within the tubes.

Results: The greatest angiogenic potential of the EMD parent was at a weight of 125 ng, resulting in a 4.3-fold increase compared with the negative control. Five pools of EMD proteins showed a stronger angiogenic activity than the EMD parent. Pool 5 showed the greatest angiogenic activity, when compared with the negative control (8.1-fold increase) and with 125 ng of the EMD parent (4.2-fold increase). Amelogenin demonstrated a significantly higher angiogenic activity than the negative control (increase up to 4.0-fold) and the EMD parent (increase up to 1.6-fold).

Conclusions: EMD parent, recombinant porcine amelogenin and certain pools of EMD proteins induced significant angiogenesis compared with the controls using a standardized in vivo assay.

Key words: amelogenin; angiogenesis; enamel matrix proteins; fibroblast growth factor; periodontal regeneration; vascular endothelial growth factor

Accepted for publication 2 November 2010

Contemporary periodontal therapy relies on the accomplishment of three main steps: the elimination of aetiologic factors, the arrest of soft and hard tissue destruction and the reformation of lost tissues. The last step involves a variety of factors and pathways responsible for the regeneration of hard (bone/cementum) and soft connective tissue (periodontal ligament) (Thoma & Cochran 2010). Various attempts have been made to regenerate periodontal tissue: (i) space-filler materials with or without biologic activity (autogenous bone, allografts, xenografts) (Nabers & O'Leary 1965, Mellonig 1991, 2000), (ii) membranes to protect the defect (guided tissue regeneration) (Nyman et al.

Conflict of interest and source of funding statement

The EMD and pools of EMD-derived proteins used in this study were manufactured and provided by Institut Straumann AG (Basel, Switzerland). Dr. Daniel Thoma was the recipient of an ITI scholarship for the period of November 2007 to October 2008 at the Department of Periodontics, University of Texas Health Science Center, at San Antonio. Dr. David L. Cochran has received consulting fees, research funding, speaking honorary and products from Institut Straumann AG. Dr. Michel Dard is an employee of Institut Straumann AG (Basel, Switzerland). All other authors do not report any conflicts of interests.

The present study was supported by an ITI Grant #506-2007 (ITI Foundation, Basel, Switzerland).

1982, Gottlow et al. 1984, 1986) and (iii) incorporation of stimulants into periodontal defects (Mellonig 1999, Nevins et al. 2005). An explanation for the often limited success of commonly used techniques aimed at regenerating the periodontal tissue may be that the therapeutic effect of a chosen technique is directed at only one tissue type and not at all the different components of the periodontal tissues. In addition, the periodontium is a highly vascularized tissue, and the failure of traditional periodontal regenerative therapies in achieving complete periodontal regeneration could be attributed to their inability to induce angiogenesis onto an avascular tooth root surface or into the newly forming tissue. The ability to exploit angiogenesis and vascularization as a therapeutic strategy is likely to provide an adequate environment for cell migration, proliferation, differentiation and extracellular matrix synthesis during the early phases of periodontal regeneration. Similarly, the therapeutic incorporation of certain biologic mediators may also specifically stimulate and coordinate regenerative processes.

An enamel matrix derivative (EMD) is currently used as a potent therapeutic agent for periodontal regeneration and soft tissue healing. The effect of EMD on periodontal regeneration has been studied in pre-clinical and clinical studies during the past few years (Hammarstrom et al. 1997, Boyan et al. 2000, Heard et al. 2000, Schwartz et al. 2000, Gutierrez et al. 2003, McGuire & Cochran 2003). Moreover, a variety of studies have analysed the basic biological processes (proliferation, chemotaxis, angiogenesis, osteopromotion, cementogenesis, dentinogenesis) initiated by EMD (Hammarstrom et al. 1997, Chong et al. 2006, Jiang et al. 2006, Zeldich et al. 2007). An EMD consists of a heterogeneous complex of polypeptides extracted from porcine enamel matrix. It is speculated that amelogenin is the primary protein responsible for the effects of EMD, as this is the most abundant protein in EMD. Accordingly, recent research has focused on the identification of bioactive pools of EMD proteins with the intent of developing a more effective material to stimulate tissue regeneration (Mumulidu et al. 2007).

Two recent reports addressed the effect of EMD on endothelial cells. EMD was found to stimulate endothelial cell proliferation and migration in vitro (Yuan et al. 2003, Schlueter et al. 2007). The major mechanism underlying the positive effects of EMD on endothelial cells is based on the ability of EMD to up-regulate vascular endothelial growth factor (VEGF) production by fibroblasts (Mirastschijski et al. 2004, Schlueter et al. 2007). Moreover, ECM contains structural proteins, such as bone sialoprotein-like proteins (Suzuki et al. 2001), that could directly stimulate the proliferation and migration of endothelial cells (Bellahcene et al. 2000). While EMD stimulates the proliferation and migration of endothelial cells, it is unclear whether EMD possesses angiogenic effects. Angiogenesis is a multistep process involving the proteolytic degradation of matrix, migration, proliferation, alignment and tube formation by vascular endothelial cells, and the maturation of newly formed vessels (Risau 1997, Yancopoulos et al. 2000, Conway et al. 2001). Therefore, additional studies are required to unequivocally demonstrate that EMD has the ability to stimulate an entire spectrum of molecular and cellular processes that result in the formation of new, functional blood vessels. For this purpose, the following steps are required: (i) to fraction EMD into smaller protein pools, (ii) to narrow down the angiogenic activity to certain protein components, (iii) to identify and further characterize pools with an increased angiogenic activity and (iv) to identify and characterize specific proteins with a high angiogenic activity. Within the present experiment, EMD was fractioned into nine pools, therefore offering the opportunity to narrow down the activity to a smaller number of proteins.

The purpose of the present study was to determine the extent to which EMD and EMD-derived proteins stimulate the development of new blood vessels (angiogenesis) in living tissues. The hypothesis was that EMD and the pools of EMD proteins stimulate angiogenesis to various degrees depending on the content and the amount of the proteins included.

Material and Methods

Separation of EMD components

EMD was separated at room temperature using size exclusion chromatography. The column used was an XK column (Pharmacia column, GE Healthcare, Buckinghamshire, UK) with a diameter of 5 cm and a length of 100 cm filled with a polyacrylamide gel (Biogel P-30 fine, Bio-Rad Laboratories, Hercules, CA, USA). The separation system included an adjunct pump (Pharmacia LKB P1, Rhys International Ltd, Manchester, UK), a collector (LKB 2211 Superrak, Pharmacia, Piscataway, NJ, USA) and a UV detector [high-pressure liquid chromatography (HPLC), UV-1575, JASCO Corporation, Tokyo, Japan] for detection. The protein was dissolved by adding a small amount of cold (6°C) 0.125 M formic acid. After loading the sample, the mobile phase used a 0.125 M formic acid with a flow rate of 60 ml/h. The complete elution of the sample required 12 h. For monitoring and purity control, the collected fractions were analysed by HPLC with a TOSOH TSK gel column (4 μm particle size, 4.6 × 300 mm). The elution method was a 9-min. isocratic run with a flow rate of 0.5 ml/min., where the mobile phase was a 20% acetonitrile, 0.9% NaCl solution and detection at 220 nm. The different fractions were then collected in nine pools (p1 to p9) and lyophilized from water. For monitoring, an SDS-PAGE (10–20%) Tris-HCl (Ready gel 10–20% Tris-HCl, Bio-Rad Laboratories) stained with Coomassie brilliant blue was performed (Fig. 1).

Animals

The current protocol was evaluated and approved by the University of Texas Health Science Center at San Antonio Institutional Use and Care of Animals Committee. The study was performed in accordance with National Institute of Health guidelines for the care and use of experimental animals.

A total number of 324 six- to eight-week-old female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN, USA) were purchased and housed in the animal care facility at the University of Texas Health Science Center at San Antonio. The animals were fed a standard diet and given free access to food and water.

Directed in vivo angiogenesis assay

In order to test the hypothesis that EMD and the pools of EMD proteins stimulate angiogenesis, a mouse model was used to quantify vessel formation and endothelial cell invasion. The angiogenic effect was analysed using the

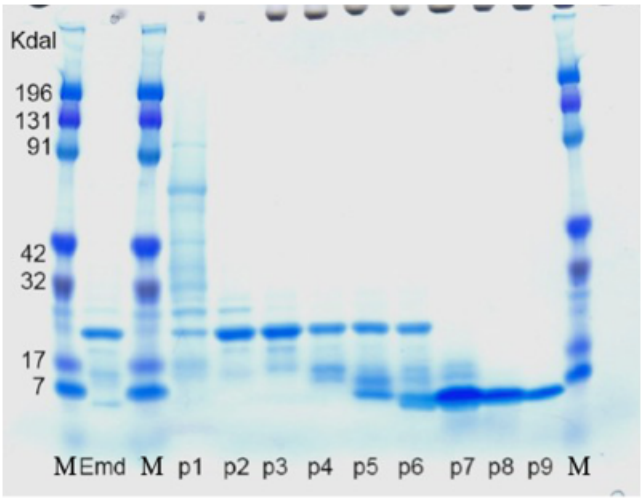

Fig. 1. Coomassie-stained SDS-PAGE gel analysis of EMD and its protein components. Lanes p1 to p9 show the chosen pools after monitoring and purity control with HPLC. Lanes marked with M contain molecular weight standards; p1–9, EMD pools 1–9; Emd, EMD parent; HPLC, high-pressure liquid chromatography; EMD, enamel matrix derivative.

commercially available Directed in Vivo Angiogenesis Assay (DIVAA™, Trevigen Inc., Gaithersburg, MD, USA), which provides a reproducible quantitative determination of angiogenesis (Guedez et al. 2003).

In brief, the mice were sedated in a chamber with 4% isoflurane in 100% O_2, and then maintained via a face mask with 1.5–2% isoflurane in 100% O_2. Following disinfection of the dorsal skin with povidone iodine and 70% isopropanol, two incisions were made on the dorsal–lateral surface of the mice, approximately 1 cm above the hip socket. The skin was pinched back and a small cut was made using dissecting scissors. The cut was extended to 1 cm in length. Implant-grade silicone cylinders closed at one end (diameter 1.5 mm; length 10 mm), called angioreactors, were filled with 20 μl of basement membrane extract (BME; continuous sheets of specialized extracellular matrix for the promotion and maintenance of differentiated cells; Trevigen Inc.) pre-mixed with or without angiogenic- or potential angiogenic-modulating factors. Two angioreactors were implanted subcutaneously in each of the two prepared pouches, resulting in four angioreactors per animal. Two angioreactors always served as controls (either a positive or a negative control); two angioreactors served as test groups in each animal. The final number of angioreactors was 16 per test group. The incisions were closed using skin staples and the mice were housed for 15 days in cages under specific mouse pathogen-free conditions.

Preparation of the reagents/applied treatment modalities for the different assays

EMD parent

To test the EMD parent (native EMD protein without the carrier; Institut Straumann AG, Basel, Switzerland), the following reagents and stock solutions were prepared:

- negative control: 200 μl BME, 11 μl sterile phosphate-buffered saline (PBS),
- positive control: 200 μl BME, 10 μl fibroblast growth factor-2 (FGF-2; 300 ng) and VEGF (100 ng), 1 μl heparin solution and
- tests 1–4: 200 μl BME, 11 μl EMD solution [0.04% acetic acid, 0.1% bovine serum albumin (BSA)]; diluted to a final weight of EMD: 500, 250, 125 or 62.5 ng per angioreactor.

EMD pools (1–9)

For testing of the nine EMD pools (Institut Straumann AG), the following reagents and stock solutions were prepared:

- negative control: 200 μl BME, 11 μl sterile PBS,
- positive control A: 200 μl BME, 10 μl FGF-2 (300 ng) and VEGF (100 ng), 1 μl heparin solution,
- positive control B: 200 μl BME, 11 μl EMD solution (3 mg/ml, 0.04% acetic acid, 0.1% BSA); diluted to a final weight of EMD: 125 ng per angioreactor and
- pools 1–9: 200 μl BME, 11 μl EMD solution pool (0.04% acetic acid, 0.1% BSA); diluted to a final weight of EMD solution pool: 10, 50 or 100 ng per angioreactor.

Comparison assays using additional control groups (FGF, VEGF, 0.25% BSA and 0.5% BSA) and purified procine amelogenin at three different weights (10, 50 and 100 ng)

The following reagents and stock solutions were prepared:

- negative control A: 200 μl BME, 11 μl sterile PBS,
- negative control B: 200 μl BME, 11 μl 0.25% BSA, diluted in 0.04% acetic acid,
- negative control C: 200 μl BME, 11 μl 0.5% BSA, diluted in 0.04% acetic acid,
- positive control A: 200 μl BME, 10 μl FGF-2 (300 ng) and VEGF (100 ng), 1 μl heparin solution,
- positive control B: 200 μl BME, 10 μl FGF-2 (300 ng; MBL International, Woburn, MA, USA), 1 μl heparin solution,
- positive control C: 200 μl BME, 10 μl VEGF (100 ng), 1 μl heparin solution,
- positive control D and E: 200 μl BME, 11 μl EMD solution (3 mg/ml; 0.04% acetic acid, 0.1% BSA); diluted to a final weight of EMD: 62.5 or 125 ng per angioreactor and
- tests 1–3: 200 μl BME, 11 μl amelogenin (0.04% acetic acid, 0.1% BSA); purified procine amelogenin (Dr. James Simmer, University of Michigan School of Dentistry, Ann Arbor, MI, USA); diluted to a final weight of EMD: 10, 50 or 100 ng per angioreactor.

Following 15 days of maintenance, 100 μl of 25 mg/ml of dextran–fluorescein isothiocyanate (dextran–FITC; Polysciences Inc., Warrington, PA, USA) in PBS was injected via the tail vein, and 20 min. later, the mice were humanely euthanized. The angioreactors were harvested using a scalpel and cut

along the open end of the angioreactors to sever any vessels. The bottom cap of the angioreactors was removed with a razor blade. The BME/vessel complex was then flushed with 300 μl of Cell-Sperse (DIVAA™, Trevigen Inc.) into a test tube. The test tubes were incubated for 1 h at 37°C, and then centrifuged for 5 min. at 15,000 *g* at room temperature in a benchtop centrifuge (Eppendorf/Brinkman, Westbury, NY, USA). Quantification of neovascularization was performed by the amount of dextran–FITC fluorescence in the supernatant. Fluorescence was measured in 96-well plates using a microplate spectrofluorometer (excitation 485 nm, emission 510 nm; HP, Perkin Elmer, Boston, MA, USA). Results were expressed in relative fluorescent units (RFUs).

Statistical analysis

The mean relative fluorescence values and standard deviations were calculated. Repeated measures were statistically analysed using ANOVA, and subsequent pairwise Student's *t*-tests with corrected *p*-values according to Bonferroni were used to detect the differences between the treatment modalities. The level of significance chosen in all the statistical tests was set at $p<0.05$. The analysis was performed using a statistical software program (GraphPad Prism, GraphPad Software, San Diego, CA, USA).

Results

All mice were healthy throughout the study and no infections or other complications related to the surgery occurred. The retrieved angioreactors were filled with newly formed tissue to various extents.

The angiogenic effects of the EMD parent, nine pools of EMD proteins and amelogenin are summarized in Table 1. Table 1 also lists the most effective weights and the fold increase of the most effective weight compared with the negative control. Overall, pool 5 showed the greatest angiogenic activity, followed by pools 3, 9, 7 and 1.

EMD parent

The results showed a dose–response curve, with a maximal angiogenic effect of the EMD parent at 125 ng per angioreactor (Fig. 2). Compared with the negative control, the positive control induced a 2.6-fold increase in the angiogenic response. The fold induction of test groups over the negative control was 1.3 (500 ng EMD parent); 1.2 (250 ng EMD parent); 4.3 (125 ng EMD parent); and 2.4 (62.5 ng EMD parent) (Fig. 2). Bonferroni's multiple comparison test showed statistically significant differences between the negative control and the positive control ($p<0.01$); the negative control and the EMD parent at 125 ng ($p<0.001$); the positive control and the EMD parent at 125 ng ($p<0.01$); and between the EMD parent at 125 ng and the two higher weights (250 and 500 ng EMD parent; $p<0.001$).

Table 1. Most effective weight of the EMD parent, all nine pools and amelogenin, as well as the increase in angiogenic activity for the most effective weight compared with the negative control for all tested groups

Group	Tested weights (ng)	Most effective weight (ng)	Increase compared with the negative control (most effective weight)
EMD parent	62.5, 125, 250, 500	125	4.3
Pool 1	10, 50, 100	50	3.4
Pool 2	10, 50, 100	50	1.7
Pool 3	10, 50, 100	10	8.2
Pool 4	10, 50, 100	10	1.2
Pool 5	10, 50, 100	10	8.1
Pool 6	10, 50, 100	10	2.3
Pool 7	10, 50, 100	50	4.7
Pool 8	10, 50, 100	10	1.6
Pool 9	10, 50, 100	50	8.1
Amelogenin	10, 50, 100	10	4.0

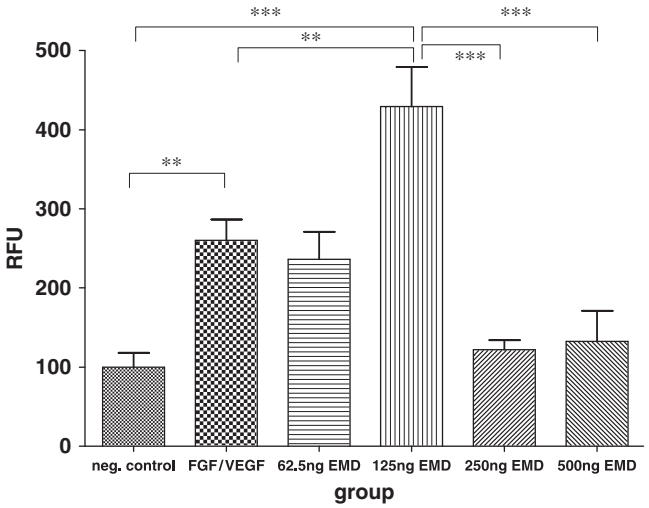

Fig. 2. Dose–response curve using four different weights of the EMD parent, a positive control (FGF/VEGF) and a negative control (BME/PBS). Bonferroni's multiple comparison test showed statistically significant differences between the negative control and the positive control FGF/VEGF ($p<0.01$); between the negative control and the EMD parent at 125 ng ($p<0.001$); between FGF/VEGF and the EMD parent at 125 ng ($p<0.01$); and between the EMD parent 125 ng and the two higher weights (250 and 500 ng EMD parent; $p<0.001$) (**$p<0.01$ and ***$p<0.001$). EMD, enamel matrix derivate; RFU, relative fluorescence unit; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; BME, basement membrane extract; PBS, phosphate-buffered saline.

EMD pool 2

The greatest angiogenic effect was observed with pool 2 at a weight of 50 ng per angioreactor (Fig. 3). The differences between pool 2 (50 ng) and the negative control ($p<0.001$) and between pool 2 at 50 ng and the other weights tested [10 ng ($p<0.01$); 100 ng ($p<0.05$)] were statistically significant. The peak values resulting from the middle weight (50 ng) of pool 2 were

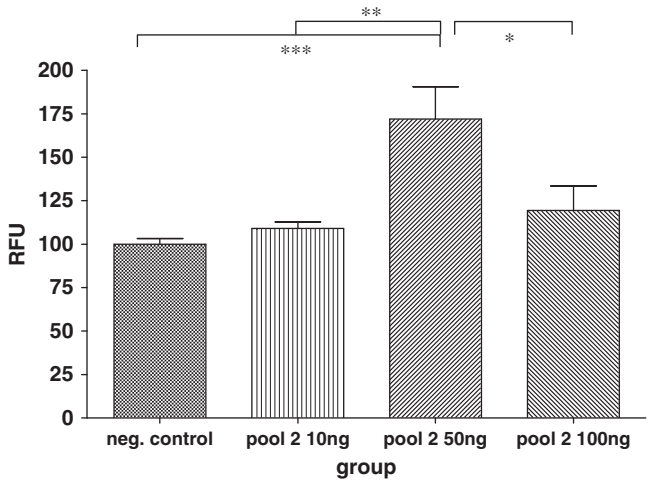

Fig. 3. Angiogenic effect between the negative control and EMD-derived pool 2 (10, 50 and 100 ng). Bonferroni's multiple comparison test revealed statistically significant differences between pool 2 at 50 ng and the following other groups: negative control ($p < 0.001$); pool 2 at 10 ng ($p < 0.01$); and pool 2 at 100 ng ($p < 0.05$). Results for positive control (FGF/VEGF) and 125 ng EMD parent are not shown ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). EMD, enamel matrix derivate; RFU, relative fluorescence unit; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

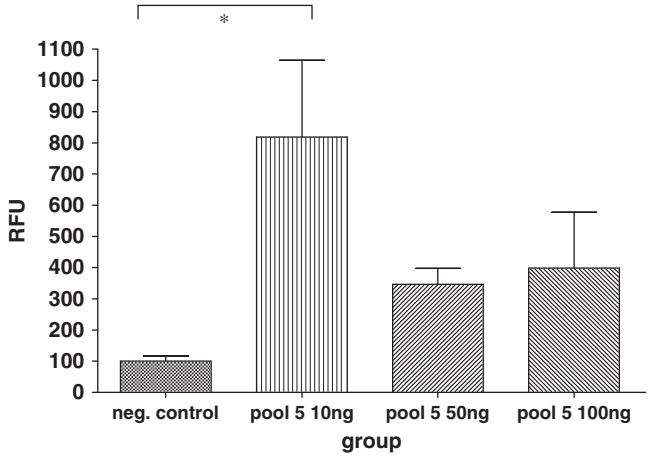

Fig. 4. Angiogenic effect between the negative control and EMD-derived pool 5 (10, 50 and 100 ng). Bonferroni's multiple comparison test revealed statistically significant differences between pool 5 at 10 ng and the negative control ($p < 0.05$). The results for the positive control (FGF/VEGF) and 125 ng EMD parent are not shown ($^{*}p < 0.05$). EMD, enamel matrix derivate; RFU, relative fluorescence unit; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

obtained using a much lower amount of protein compared with the EMD parent. EMD pool 2 induced a 1.1-fold (10 ng pool 2), 1.7-fold (50 ng) and 1.2-fold (100 ng) increase in the angiogenic effect compared with the negative control.

EMD pool 5

EMD pool 5 significantly stimulated in vivo angiogenesis. The strongest effects were observed with the lower concentration used in this assay (10 ng per angioreactor). Statistically significant differences were observed using 10 ng of pool 5 compared with the negative control ($p < 0.05$). EMD pool 5 concentrations revealed an 8.1-fold (10 ng), a 3.5-fold (50 ng) and a 3.8-fold (100 ng) increase in the angiogenic effect compared with the negative control (Fig. 4).

EMD pool 9

The greatest increase in angiogenic activity was observed using the lower (10 ng) and middle (50 ng) weights of pool 9 (Fig. 5). Statistically significant differences were found between pool 9 (50 ng) and the negative control ($p < 0.01$), and between pool 9 at an intermediate (50 ng) and a higher weight (100 ng) ($p < 0.01$). The increase between the three different concentrations of pool 9 compared with the negative control amounted to 8.1-fold (10 ng), 11-fold (50 ng) and 1.6-fold (100 ng).

Comparison assays using additional control groups and purified porcine amelogenin at three different concentrations

To exclude the possibility that protein aggregation could induce angiogenesis, additional experiments using BSA were performed. The two concentrations used (0.25% BSA and 0.5% BSA) resulted in mean values comparable to the standard negative control group without any statistically significant differences. The mean RFU values of VEGF alone and FGF alone were comparable to the combination of FGF/VEGF. Again, no statistically significant differences were observed (Table 2).

As amelogenin comprises 90% of the protein content in EMD, the angiogenic potential of amelogenin was investigated. A dose–response curve was observed using amelogenin, with the greatest mean RFU values using the lowest weight (10 ng). However, the differences between the three tested weights were not statistically significant. Bonferroni's multiple comparison test showed statistically significant differences between amelogenin at 10 ng and the negative control ($p < 0.001$), FGF/VEGF ($p < 0.01$), VEGF alone ($p < 0.01$), FGF alone ($p < 0.01$), EMD parent at 62.5 ng ($p < 0.01$), 0.25% BSA ($p < 0.05$) and 0.5% BSA ($p < 0.01$). Comparisons between amelogenin at 50 ng and the other groups resulted in statistically significant differences [negative control ($p < 0.01$), VEGF alone ($p < 0.05$), FGF alone ($p < 0.05$) and 0.5% BSA ($p < 0.05$)]. Comparable to previous assays, the use of EMD parent at 125 ng resulted in greater mean values than at 62.5 ng (Table 2).

Discussion

Periodontal regeneration relies on the reformation of lost hard and soft tissues and, specifically, on the stimulation of hard and soft tissue cells during the three stages of the wound-healing process: (i)

inflammatory phase, (ii) tissue formation phase and (iii) tissue remodelling phase (Singer & Clark 1999, Thoma & Cochran 2010). Angiogenesis has been accepted as one of the major determinants during the wound-healing process (Tonnesen et al. 2000). As the periodontium is a highly vascularized tissue, the inability to induce angiogenesis might explain a failure in achieving complete periodontal regeneration. Current therapeutic regeneration approaches include the use of biologic mediators (e.g. EMD) as an adjunctive aide. As EMD contains a combination of proteins, the potential exists for multiple biological effects as it has been demonstrated in a number of studies (Hammarstrom et al. 1997, Boyan et al. 2000, Heard et al. 2000, Schwartz et al. 2000, Gutierrez et al. 2003, McGuire & Cochran 2003). These proteins positively influence periodontal wound regeneration by modulating periodontal ligament cells, cementum cells, bone cells, gingival fibroblasts and endothelial cells. These pre-clinical and clinical studies demonstrated that EMD creates an environment favourable for periodontal regeneration. However, the underlying mechanism of the regenerative process is not fully understood. Recent research focused therefore on the various specific activities of EMD and EMD-derived proteins.

It has been suggested previously that EMD enhances new blood vessel formation in a murine model. Microscopic findings showed significantly more blood vessels surrounding collagen implants combined with EMD *versus* without EMD in mice (Yuan et al. 2003). Using a different animal model, our study confirmed the angiogenic effect of EMD. Moreover, our results are also consistent with a recent in vivo study that demonstrated the ability of EMD to induce the formation of new blood vessels in the chorioallantoic membrane (CAM) of developing chicken eggs (Kauvar et al. 2010).

Various concentrations of EMD were tested in the present study and the results demonstrated that EMD stimulated angiogenesis in a dose-dependent manner. The most effective weight for the EMD parent was 125 ng, resulting in a 4.3-fold increase in angiogenesis as compared with the negative control. While a 50% lower dose (62.5 ng) induced a 2.4 fold increase in angiogenesis as compared with the negative control, no benefits with respect to new blood vessel formation were observed with two higher weights (250 and 500 ng). The observation that higher concentrations of EMD are less effective is well known and has been reported previously for angiogenesis and for bone regeneration (Plachokova et al. 2008, Kauvar et al. 2010).

EMD is an extract of low-molecular-weight porcine enamel proteins, mainly consisting of amelogenin. Accordingly, EMD activity has been attributed predominantly to amelogenin. However, it is unclear whether other proteins are involved in the various biological effects (e.g. angiogenesis) induced by the use of EMD as demonstrated in in vitro, pre-clinical and clinical studies (Cochran et al. 2003, McGuire & Cochran 2003, Schlueter et al. 2007). It is reasonable to assume that the multiple biological functions of EMD may be attributed to specific proteins found in the heterogeneous complex of EMD. In order to investigate the angiogenic effect of EMD proteins, pools of EMD proteins were separated and thereafter screened and tested using an in vivo model. Our results demonstrated that some EMD pools induced angiogenesis in a dose-dependent manner. Two pools (3 and 5) showed the greatest angiogenic effect at a weight of 10 ng, whereas pools 7, 9 and 1 were the most effective at 50 ng. Overall, pool 5

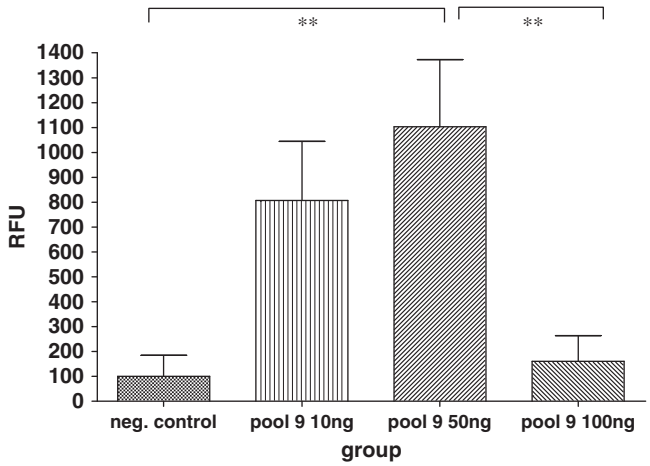

Fig. 5. Angiogenic effect between negative control and EMD-derived pool 9 (10, 50 and 100 ng). Bonferroni's multiple comparison test revealed statistically significant differences between pool 9 at 50 ng and the negative control ($p < 0.01$) and between pool 2 at 50 ng and pool 2 at 100 ng ($p < 0.01$). The results for positive control (FGF/VEGF) and 125 ng EMD parent are not shown ($^{**}p < 0.01$). EMD, enamel matrix derivate; RFU, relative fluorescence unit; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

Table 2. Mean values and lower and upper 95% confidence interval (CI) for the control groups (negative; FGF, FGF/VEGF, VEGF) and the EMD parent at 62.5 ng, EMD parent at 125 ng, two concentrations of BSA, and amelogenin at three weights, as well as the increase in angiogenic activity for the test and control groups compared with the negative control

Groups	Controls				EMD parent		BSA		Amelogenin		
	negative control	FGF/VEGF	VEGF	FGF	62.5 ng	125 ng	0.25%	0.5%	10 ng	50 ng	100 ng
Mean	100.0	169.8	159.7	150.9	179.9	246.4	116.1	122.7	402.1	354.5	331.9
Lower 95% CI	78.54	138.6	115.4	126.1	142.7	170.8	34.40	97.26	230.0	255.1	263.3
Upper 95% CI	121.5	201.0	204.0	175.6	217.1	322.0	197.7	148.1	574.1	453.8	400.6
Fold increase compared with the negative control	NA	1.7	1.6	1.5	1.8	2.5	1.2	1.2	4.0	3.5	3.3

FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; EMD, enamel matrix derivate.

showed the greatest angiogenic activity, followed by pools 3, 9, 7 and 1. In addition, these pools (1, 3, 5, 7 and 9) showed a stronger angiogenic activity as compared with the EMD parent, indicating an enrichment of the active protein(s) or the removal of an inhibitor factor. Several reasons may explain the more effective angiogenesis obtained using some of the separated proteins (EMD pools): (i) the concentrations of EMD proteins (i.e. pools 1, 3, 5, 7 and 9) were optimized for an angiogenic effect in comparison with the EMD parent, (ii) two or more proteins may have a synergistic effect but the effect is obscured by all the proteins in the native mixture and (iii) inhibitory proteins may be present that limit angiogenic activity. The dose–response curve when using the EMD parent clearly demonstrated that higher doses do not have an angiogenic effect, whereas lower concentrations increased new blood vessel formation. Accordingly, the concentrations used for the EMD pools were evaluated in smaller amounts. This resulted in some of the EMD pools demonstrating strong angiogenic effects. It may be speculated that the optimal weight might even be lower than the ones tested as the tested pools likely still consisted of multiple proteins. The present results suggest that the angiogenic activity of native EMD is associated with either more than one protein or one protein and its fragments as several of the pools showed new blood vessel formation. These findings are supported by the results of the SDS-PAGE gel analysis. Three main proteins fractions can be identified in pool 5 (the 25 kDa amelogenin plus a 7 and a 5 kDa protein). Amelogenin may also be present in pool 3 (the second most effective pool), together with some breakdown in the 20 and 12 kDa region. Pools 7 and 9 may contain at least the 5 kDa protein, yielding some intermediate activity. Therefore, it can be assumed that not only amelogenin has an angiogenic activity but also at least the 5 kDa protein. The combination of at least two proteins led to a potent angiogenic activity. One could also speculate that there is an inhibitory effect of one or more proteins. In some of the pools, the inhibitory effect may have covered the angiogenic response, whereas in pools without the inhibitory protein, increased blood vessel formation occurred. Based on these results, it is assumed that even though EMD has a strong angiogenic effect, the separation of EMD proteins may allow for optimizing angiogenic activity.

Earlier studies attempted to attribute the effects of EMD to specific proteins (for a review, see Foster et al. 2007). Both EMD and amelogenin have been shown to accelerate wound closure of full-thickness skin wounds in rabbits by stimulating fibroblast proliferation and early wound contraction (Rincon et al. 2003, Mirastschijski et al. 2004, Grayson et al. 2006). These studies suggest that amelogenin could be the protein responsible for the positive effects of EMD. Amelogenin has also been tested for new blood vessel formation in various studies, with mixed results (Schlueter et al. 2007, Johnson et al. 2009, Kauvar et al. 2010). Two in vitro studies reported an angiogenic response of recombinant amelogenin greater than the negative control, but failed to show any statistical significance (Schlueter et al. 2007, Johnson et al. 2009). In contrast, an in vivo study evaluating recombinant human amelogenin in the CAM assay demonstrated that amelogenin was indeed angiogenic with histologically assessed new blood vessel formation comparable to the positive control (FGF/VEGF) (Kauvar et al. 2010). The differences in the angiogenic response between the studies may be due to the experimental design (in vitro *versus* in vivo) and/or due to the species differences used in the respective experiments (recombinant porcine *versus* recombinant human amelogenin). The results from the present study are consistent with previous in vivo data suggesting that amelogenin is a strong and probably the most effective angiogenic factor of EMD. Although amelogenin outperformed the EMD parent proteins, it was still less effective than two pools (i.e. pools 5 and 7) in its ability to induce new blood vessel formation. Because the proteins in each of the pools have not been characterized or identified, it is not possible to know whether several proteins or one protein and its breakdown products are responsible for the observed angiogenic effects. Indeed, it is also possible that EMD contains both inducers and inhibitors of angiogenesis, and that a combination of inducer EMD proteins at an optimal ratio without inhibiting factors may have the most potent effect on angiogenesis. Future research will be directed to further characterize and evaluate the angiogenic activity of EMD proteins included in the most potent pools (i.e. pools 5 and 7), eventually identifying specific proteins or protein fragments (at specific weights) with the greatest angiogenic potential, but also the ones, if they exist, with an inhibitory effect.

Conclusions

The present in vivo study demonstrated that the use of EMD and protein pools derived from the parent EMD results in an angiogenic response to various extents. The greatest angiogenic potential of the EMD parent was observed at a weight of 125 ng and resulted in a 4.3-fold increase in comparison with the negative control. The use of the nine pools derived from the parent EMD revealed differences with respect to the ability to stimulate new blood vessel formation. Some pools did not show any angiogenic effect, whereas others were responsible for a potent angiogenic activity at the lowest tested weight (10 ng). Overall, pool 5 showed the greatest angiogenic activity when compared with the negative control (8.1-fold increase) and with the 125 ng EMD parent (4.2-fold increase). The results suggest that the angiogenic activity of EMD may be attributed to one protein and its fragments or to multiple proteins and that both inhibitors and inducers of angiogenesis may be present in the parent–EMD mixture of proteins.

Acknowledgements

The authors would like to thank Dr. James Simmer (University of Michigan School of Dentistry, Ann Arbor, MI, USA) for generously providing us with recombinant porcine amelogenin.

References

Bellahcene, A., Bonjean, K., Fohr, B., Fedarko, N. S., Robey, F. A., Young, M. F., Fisher, L. W. & Castronovo, V. (2000) Bone sialoprotein mediates human endothelial cell attachment and migration and promotes angiogenesis. *Circulation Research* **86**, 885–891.

Boyan, B. D., Weesner, T. C., Lohmann, C. H., Andreacchio, D., Carnes, D. L., Dean, D. D., Cochran, D. L. & Schwartz, Z. (2000) Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. *Journal of Periodontology* **71**, 1278–1286.

Chong, C. H., Carnes, D. L., Moritz, A. J., Oates, T., Ryu, O. H., Simmer, J. & Cochran, D. L. (2006) Human periodontal fibroblast response to enamel matrix derivative, amelogenin, and platelet-derived growth factor-BB. *Journal of Periodontology* **77**, 1242–1252.

Cochran, D. L., Jones, A., Heijl, L., Mellonig, J. T., Schoolfield, J. & King, G. N. (2003) Periodontal regeneration with a combination of enamel matrix proteins and autogenous bone grafting. *Journal of Periodontology* **74**, 1269–1281.

Conway, E. M., Collen, D. & Carmeliet, P. (2001) Molecular mechanisms of blood vessel growth. *Cardiovascular Research* **49**, 507–521.

Foster, B. L., Popowics, T. E., Fong, H. K. & Somerman, M. J. (2007) Advances in defining regulators of cementum development and periodontal regeneration. *Current Topics in Developmental Biology* **78**, 47–126.

Gottlow, J., Nyman, S., Karring, T. & Lindhe, J. (1984) New attachment formation as the result of controlled tissue regeneration. *Journal of Clinical Periodontology* **11**, 494–503.

Gottlow, J., Nyman, S., Lindhe, J., Karring, T. & Wennstrom, J. (1986) New attachment formation in the human periodontium by guided tissue regeneration. Case reports. *Journal of Clinical Periodontology* **13**, 604–616.

Grayson, R. E., Yamakoshi, Y., Wood, E. J. & Agren, M. S. (2006) The effect of the amelogenin fraction of enamel matrix proteins on fibroblast-mediated collagen matrix reorganization. *Biomaterials* **27**, 2926–2933.

Guedez, L., Rivera, A. M., Salloum, R., Miller, M. L., Diegmueller, J. J., Bungay, P. M. & Stetler-Stevenson, W. G. (2003) Quantitative assessment of angiogenic responses by the directed in vivo angiogenesis assay. *American Journal of Pathology* **162**, 1431–1439.

Gutierrez, M. A., Mellonig, J. T. & Cochran, D. L. (2003) Evaluation of enamel matrix derivative as an adjunct to non-surgical periodontal therapy. *Journal of Clinical Periodontology* **30**, 739–745.

Hammarstrom, L., Heijl, L. & Gestrelius, S. (1997) Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. *Journal of Clinical Periodontology* **24**, 669–677.

Heard, R. H., Mellonig, J. T., Brunsvold, M. A., Lasho, D. J., Meffert, R. M. & Cochran, D. L. (2000) Clinical evaluation of wound healing following multiple exposures to enamel matrix protein derivative in the treatment of intrabony periodontal defects. *Journal of Periodontology* **71**, 1715–1721.

Jiang, J., Goodarzi, G., He, J., Li, H., Safavi, K. E., Spangberg, L. S. & Zhu, Q. (2006) Emdogain-gel stimulates proliferation of odontoblasts and osteoblasts. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **102**, 698–702.

Johnson, D. L., Carnes, D. L., Steffensen, B. & Cochran, D. L. (2009) Cellular effects of enamel matrix derivative are associated with different molecular weight fractions following separation by size-exclusion chromatography. *Journal of Periodontology* **80**, 648–656.

Kauvar, A. S., Thoma, D. S., Carnes, D. L. & Cochran, D. L. (2010) In vivo angiogenic activity of enamel matrix derivative. *Journal of Periodontology* **81**, 1196–1201.

McGuire, M. K. & Cochran, D. L. (2003) Evaluation of human recession defects treated with coronally advanced flaps and either enamel matrix derivative or connective tissue. Part 2: histological evaluation. *Journal of Periodontology* **74**, 1126–1135.

Mellonig, J. T. (1991) Freeze-dried bone allografts in periodontal reconstructive surgery. *Dental Clinics of North America* **35**, 505–520.

Mellonig, J. T. (1999) Enamel matrix derivative for periodontal reconstructive surgery: technique and clinical and histologic case report. *International Journal of Periodontics & Restorative Dentistry* **19**, 8–19.

Mellonig, J. T. (2000) Human histologic evaluation of a bovine-derived bone xenograft in the treatment of periodontal osseous defects. *International Journal of Periodontics & Restorative Dentistry* **20**, 19–29.

Mirastschijski, U., Konrad, D., Lundberg, E., Lyngstadaas, S. P., Jorgensen, L. N. & Agren, M. S. (2004) Effects of a topical enamel matrix derivative on skin wound healing. *Wound Repair and Regeneration* **12**, 100–108.

Mumulidu, A., Hildebrand, B., Fabi, B., Hammarstrom, L., Cochran, D. L., Dard, M. & Lemoult, S. (2007) Purification and analysis of a 5 kDa component of enamel matrix derivative. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* **857**, 210–218.

Nabers, C. L. & O'Leary, T. J. (1965) Autogenous bone transplants in the treatment of osseous defects. *Journal of Periodontology* **36**, 5–14.

Nevins, M., Giannobile, W. V., McGuire, M. K., Kao, R. T., Mellonig, J. T., Hinrichs, J. E., McAllister, B. S., Murphy, K. S., McClain, P. K., Nevins, M. L., Paquette, D. W., Han, T. J., Reddy, M. S., Lavin, P. T., Genco, R. J. & Lynch, S. E. (2005) Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomized controlled trial. *Journal of Periodontology* **76**, 2205–2215.

Nyman, S., Gottlow, J., Karring, T. & Lindhe, J. (1982) The regenerative potential of the periodontal ligament. An experimental study in the monkey. *Journal of Clinical Periodontology* **9**, 257–265.

Plachokova, A. S., van den Dolder, J. & Jansen, J. A. (2008) The bone-regenerative properties of Emdogain adsorbed onto poly(D, L-lactic-coglycolic acid)/calcium phosphate composites in an ectopic and an orthotopic rat model. *Journal of Periodontal Research* **43**, 55–63.

Rincon, J. C., Haase, H. R. & Bartold, P. M. (2003) Effect of Emdogain on human periodontal fibroblasts in an in vitro wound-healing model. *Journal of Periodontal Research* **38**, 290–295.

Risau, W. (1997) Mechanisms of angiogenesis. *Nature* **386**, 671–674.

Schlueter, S. R., Carnes, D. L. & Cochran, D. L. (2007) In vitro effects of enamel matrix derivative on microvascular cells. *Journal of Periodontology* **78**, 141–151.

Schwartz, Z., Carnes, D. L. Jr., Pulliam, R., Lohmann, C. H., Sylvia, V. L., Liu, Y., Dean, D. D., Cochran, D. L. & Boyan, B. D. (2000) Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. *Journal of Periodontology* **71**, 1287–1296.

Singer, A. J. & Clark, R. A. (1999) Cutaneous wound healing. *New England Journal of Medicine* **341**, 738–746.

Suzuki, N., Ohyama, M., Maeno, M., Ito, K. & Otsuka, K. (2001) Attachment of human periodontal ligament cells to enamel matrix-derived protein is mediated via interaction between BSP-like molecules and integrin alpha(v)beta3. *Journal of Periodontology* **72**, 1520–1526.

Thoma, D. S. & Cochran, D. L. (2010) Unlocking the mystery of periodontal regeneration – different tissues, different phenotypes. *Journal de Parodontologie et d'Implantologie Orale* **17**, 23–60.

Tonnesen, M. G., Feng, X. & Clark, R. A. (2000) Angiogenesis in wound healing. *Journal of Investigative Dermatology. Symposium Proceedings* **5**, 40–46.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J. & Holash, J. (2000) Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242–248.

Yuan, K., Chen, C. L. & Lin, M. T. (2003) Enamel matrix derivative exhibits angiogenic effect in vitro and in a murine model. *Journal of Clinical Periodontology* **30**, 732–738.

Zeldich, E., Koren, R., Nemcovsky, C. & Weinreb, M. (2007) Enamel matrix derivative stimulates human gingival fibroblast proliferation via ERK. *Journal of Dental Research* **86**, 41–46.

Address:
Daniel S. Thoma
Clinic for Fixed and Removable Prosthodontics and Dental Material Science
University of Zurich
Plattenstrasse 11
CH-8032 Zurich
Switzerland
E-mail: daniel.thoma@zzmk.uzh.ch

Clinical relevance

Scientific rationale for the study: EMD has been shown to regulate a variety of processes during periodontal wound healing, among these, the induction of angiogenesis. EMD consists of a heterogeneous complex of polypeptides. It is unknown whether all of these proteins or only a specific protein and/or breakdown products are responsible for the angiogenic activity.

Principal findings: EMD and certain pools of EMD proteins induced significant angiogenesis in an in vivo model. Based on the results of this in vivo assay, several proteins or one protein and its breakdown products may be responsible for the angiogenic activity.

Practical implications: It is of interest to characterize the EMD components and/or their breakdown products to determine which proteins or protein fragments are responsible for the angiogenic activity associated with EMD.