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Effect of different enamel matrix derivative proteins on behavior and differentiation of endothelial cells

Oleh Andrukhov[a,f,*], Anja C. Gemperli[b], Yan Tang[a,c], Nadia Howald[b], Michel Dard[b,d], Frank Falkensammer[e], Andreas Moritz[f], Xiaohui Rausch-Fan[a,f,*]

[a] Competence Centre of Periodontal Research, Bernhard Gottlieb School of Dentistry, Medical University of Vienna, Austria
[b] Institut Straumann AG, Basel, Switzerland
[c] Department of Stomatology, Xuanwu Hospital, Capital Medical University, Beijing, China
[d] New York University, College of Dentistry, Department of Periodontology and Implant Dentistry, New York, NY, USA
[e] Division of Orthodontics, Bernhard Gottlieb School of Dentistry, Medical University of Vienna, Vienna, Austria
[f] Division of Conservative Dentistry, Periodontology and Prophylaxis, Bernhard Gottlieb School of Dentistry, Medical University of Vienna, Vienna, Austria

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ABSTRACT

Objectives. Enamel matrix derivative (EMD) is an effective biomaterial for periodontal tissue regeneration and might stimulate angiogenesis. In order to clarify mechanisms underlying its biological activity, we separated two EMD fractions with different molecular weight protein components and investigated their effects on human umbilical vein endothelial cells (HUVECs) *in vitro*.

Methods. Fraction Low-Molecular Weight (LMW) included proteins with a molecular weight (M.W.) < 8 kDa. Fraction LMW-depleted included proteins with M.W. > 8 kDa and lower than approximately 55 kDa. The effect of EMD fractions on proliferation/viability, apoptosis, migration and expression of angiopoetin-2 (ang-2), von Willebrand factor (vWF), E-selectin, intracellular adhesion molecules 1 (ICAM-1), vascular endothelial growth factor (VEGF) receptors Flt-1 and KDR was investigated.

Results. The proliferation/viability of HUVECs was inhibited by both LMW and LMW-depleted at concentrations 100 μg/ml, whereas EMD slightly increased cell proliferation/viability. The expression of all investigated proteins was up-regulated by EMD. However, differences in the effect of EMD fractions on the protein expression were observed. The effect of LMW-depleted on the expression of ICAM-1 and E-selectin was markedly higher compared to LMW. In contrast, the expression of vWF and VEGF receptors Flt-1 and KDR was primarily affected LMW than by LMW depleted. The expression of ang-2 was not influenced by LMW and LMW-depleted. HUVECs migration was stimulated more strongly by LMW than by EMD and LMW-depleted.

* *Corresponding authors at*: Bernhard Gottlieb School of Dentistry, Medical University of Vienna, Sensengasse 2a, A-1090 Vienna, Austria. Tel.: +43 1 40070 2620/+43 1 40070 4748; fax: +43 1400702609.
E-mail addresses: oleh.andrukhov@meduniwien.ac.at (O. Andrukhov), xiaohui.rausch-fan@meduniwien.ac.at (X. Rausch-Fan).

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Conclusion. Our *in vitro* study shows that the proteins composing EMD have different and specific biological activities and consequently have the ability to cover different aspects of EMD's biological and clinical effects.

1. Introduction

Healing of periodontal tissue is a complex process, which involves formation of tissues due to the complex interaction of several types of cells [1]. Application of bioactive material is considered an important approach to improve the regeneration of periodontal tissue. Enamel matrix derivative (EMD) is a complex of low-molecular weight hydrophobic enamel proteins, which is derived from developing porcine tooth buds. The EMD-based commercial product Emdogain®, which contains also a propylene glycol alginate (PGA) carrier, has been used clinically since more than 10 years, and its capacity to promote periodontal regeneration has been largely documented [2,3]. The influence of EMD on biological processes seems to be based to the presence of bioactive compounds, which mimic the process of teeth development [4,5].

Angiogenesis is an important process involved in the periodontal regeneration and wound healing [6]. Periodontium is a highly vascularised tissue and therefore success of therapy depends on the ability to promote the formation of blood microvessels, which guarantee nutrition and oxygen supply. Endothelial cells (ECs), which underlie the inner surface of the vasculature, play a key role in angiogenesis. The process of new vessel formation includes sprouting of ECs from the existing vessel, proliferation, migration, and organization in the capillary network [7]. An ability of EMD to stimulate angiogenesis *in vivo* is observed by both clinical and animal studies [8,9]. *In vitro* studies show that EMD stimulates ECs migration, *in vitro* angiogenesis, and expression of angiogenesis-related proteins [10–13]. Finally, it is demonstrated in a recent study that EMD stimulates angiogenic differentiation of periodontal ligament derived stem cells [14].

Studies in recent years focus on the identification of specific components in EMD responsible for its biological activity. EMD is composed mainly of amelogenin and amelogenin transcripts resulting from gene alternative splicing [15,16]. Size exclusion chromatography shows that EMD contains three major protein fractions with molecular weight of 20, 9 + 12, and 5 kDa [17]. Fraction 20 kDa is thought to represent whole length amelogenin, whereas fractions 9 + 12 kDa and 5 kDa seems to contain leucine-rich amelogenin peptide (LRAP) and tyrosine-rich amelogenine peptide (TRAP), respectively [4]. Several previous studies investigated the effect of different EMD fractions on angiogenesis [9,18–20], but the results of these studies are partially controversial. Particularly, some studies suggest an angiogenic activity of 5 kDa EMD protein [9,20], whereas another study reports no effect of the 5 kDa EMD protein on blood vessel formation in the chorioallantoic membrane of the developing chicken eggs [19]. Johnson et al. suggest that the cellular activity of EMD was not associated with a single molecular weight species and the effects of EMD on proliferation and angiogenesis process depends on the presence of several low molecular weight proteins [18].

In order to further characterize the angiogenic potential of different EMD protein, we investigated their effect on endothelial cells *in vitro*. Two fractions of proteins were separated from EMD: fraction low molecular weight (LMW)-depleted included proteins with a molecular weight of 8–55 kDa and presumably containing whole length amelogenin and LRAP; fraction LMW included proteins with a molecular weight less than 8 kDa and presumably mostly composed of TRAP. The effect of these fractions on the proliferation/viability, apoptosis, migration, and differentiation of HUVECs was analyzed *in vitro*. The expression of several proteins involved in wound healing and angiogenesis was examined: ang-2, E-selectin, ICAM-1, von Willebrand factor (vWF), vascular endothelial growth factor (VEGF) receptor-1 (Flt-1), and VEGF receptor-2 (KDR).

2. Material and methods

2.1. Cells and materials

Commercially available HUVECs pooled from 10 different healthy donors (Technoclone, Vienna, Austria) were used in the present study. HUVECs were cultured in endothelial cell medium (ECM, Technoclone, Austria) with 20% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 2 mM L-glutamine, 5 U/ml heparin and 30–50 μg/ml endothelial cell growth supplement in culture flasks coated with 0.2% gelatine at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The HUVECs from the 4th to 7th passage in culture were used.

2.2. EMD separation

EMD fractions LMW and LMW-depleted were separated and purified by Institut Straumann using a modification of previously described methods [17,21]. Briefly, EMD fractions LMW and LMW-depleted were extracted from porcine enamel matrix derivative via size exclusion high-performance liquid chromatography (Shodex KW 2003, Brechbühler AG, Switzerlandy) in 100 mM Na acetate pH 3.5 containing 100 mM NaCl. Lyophilized fractions were reconstituted in 0.1% acetic acid to produce a 10 mg/ml stock solution. Further dilutions of proteins (1–100 μg/ml) were performed into FBS-free ECM.

The working solution of 100 μg/ml EMD or EMD fractions contained 0.001% of acetic acid, which did not exert any significant effect on any parameter investigated in this study.