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Effect of enamel matrix derivative on the angiogenic behaviors of human umbilical vein endothelial cells on different titanium surfaces

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Angiogenesis play a crucial role in the regeneration of hard and soft tissue around dental titanium (Ti) implant. Enamel matrix derivative (EMD) promotes tissue regeneration and stimulates angiogenesis but its effect on the angiogenesis on Ti surfaces was never investigated. The effect of EMD on the angiogenic activity of endothelial cells cultured on pre-treated smooth Ti (PT), acid-etched (A), coarse-grit blasted and acid-etched (SLA) surfaces and tissue culture plastic (TCP) in the presence or absence of EMD was investigated. EMD inhibited the proliferation/viability of human umbilical vein endothelial cells (HUVECs) growing on A and SLA Ti surfaces. EMD induced an increase in the expression of all these genes in HUVECs grown on SLA surface but not on other surfaces. Summarizing, our data show that EMD influences proliferation and expression of angiogenesis associated gene in HUVECs grown on moderately rough SLA surfaces, suggesting that EMD might promote angiogenesis following implantation.

Keywords: Endothelial cells, Dental implants, Titanium surface, Surface roughness, Enamel matrix derivative

INTRODUCTION

Implant osseointegration is largely influenced by the properties of Ti surface[1]. *In vitro* studies show that osteogenic differentiation of osteoblast and mesenchymal stem cells is promoted by Ti surface with moderate micron scale roughness, hydrophilic surfaces, and surfaces containing nanostructures[2-5]. Angiogenesis or formation of new blood vessels is an important process, which crucially influences the process of bone healing and tissue regeneration around dental implant[6]. Although there is no direct contact between blood vessels and implant surface in osseointegrated implant, appropriate blood supply plays an important role in healing of both hard and soft tissues. Studies of the last years focus on investigating the role of angiogenesis and the interaction between angiogenesis and osteogenesis in wound healing after implantation[7-9]. Endothelial cells (ECs), which underlie the inner surface of the vasculature, play a key role in the angiogenesis, and therefore their interaction with titanium surfaces is an important factor influencing tissue healing.

Moderately rough Ti surfaces seem to be not optimal for endothelial cells and might have a negative effect on angiogenesis[9,10]. The negative effect of these surfaces on angiogenesis might be theoretically compensated by using of biologically active substances. Enamel matrix derivative (EMD) is a complex of low-molecular weight hydrophobic enamel proteins isolated from developing porcine tooth buds and consists by approximately 90% of amelogenin. An ability of EMD to stimulate angiogenesis *in vivo* was shown by both clinical and animal studies[11,12]. *In vitro* studies show that EMD possesses chemotactic ability, stimulates ECs migration, formation of vessel like structures, and increase the expression of angiogenesis-related genes[13-16]. Some studies suggest that the effect of EMD on angiogenesis is biphasic: Angiogenic properties of endothelial cells seem to be promoted by moderate EMD concentrations and inhibited by higher EMD concentrations[16]. We assumed that EMD might counterweigh the negative effect of moderately rough Ti surface on endothelial cells function and investigated the effects of EMD on the proliferation and differentiation of EC on Ti surfaces with different micron scale roughness.

MATERIALS AND METHODS

Test titanium disks were prepared from 1-mm-thick sheets of grade 2 unalloyed commercially pure titanium (Institut Straumann, Basel, Switzerland) and were punched to be 15 mm in diameter to fit into the well of a 24-well tissue culture plate. Pickled (PT) disks were degreased by washing in acetone and processed through a 2% ammonium fluoride, 2% hydrofluoric acid, 10% nitric acid solution at 55°C for 30 s. The acid-etched (A) surface and coarse-grit acid-etched surfaces (SLA) were prepared as described previously[3,9]. Arithmetic mean deviation of the surface (Sa) parameter was 0.60±0.10 μm for PT surface, 0.60±0.01 μm for A surface, and 1.20±0.03 μm for SLA surface.

Commercially available human umbilical vein endothelial cells (HUVECs, Technoclone, Vienna, Austria) from the passage 4-7 in culture were used.

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HUVECs were cultured in endothelial cell growth medium (ECM, Technoclone). The cell culture flasks were precoated with 0.2% gelatine.

PT, A and SLA surfaces were were firstly placed in 24-well plates, then 500 μL HUVEC suspension with 1×10^5 cells were pipetted into each cell culture well. After 24 h of culture at 37°C, the culture medium in the Ti surface groups was replaced with 500 μL of ECM containing EMD at a concentration of 50 μg/mL. The EMD powder was dissolved in 0.1% acetic acid and further diluted in ECM to the final working concentration of 50 μg/mL. The culture medium in the control TCPS group and the Ti surface groups without EMD were replaced with 500 μL of ECM containing 0.0005% acetic acid[10,16,17].

The number of HUVECs cultured on different surfaces with and without EMD was evaluated by manual cell counting. Proliferation/viability of HUVECs in all experimental groups was evaluated with the MTT assay[18].

HUVECs were seeded on different Ti surface at a density of 1×10^5 cells/well and cultured for 24 h in ECM. Three samples from each group were randomly chosen to evaluate the morphology of HUVECs on different Ti surfaces. After 24 culturing, the samples were rinsed with PBS to remove unattached cells. Then the samples were fixed with 4% formalin for 24 h and dehydrated with different concentration ethanol and Hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO, USA). Finally, the samples were spattered with gold for 3 min. The cells were observed by a scanning electron microscopy (FEI Quanta 200) at an accelerating voltage of 20 kV.

The expression of the angiogenesis related genes intercellular adhesion molecule-1 (ICAM-1), E-selectin, endothelial protein C receptor (EPCR), von Willebrand Factor (vWF), and angopoietin-2 (Ang-2) in HUVECs was measured by quantitative polymerase chain reaction (qPCR) as described previously[13,19] using TaqMan®Gene Expression Cells-to-CT™ kit and following primers (all from Applied Biosystems, Foster City, CA, USA): vICAM-1, Hs00164932_m1; E-selectin, Hs00950401_m1; EPCR, Hs00941182_m1; WF, Hs00169795_m1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs99999905_m1. Triplicate PCR thermocycling was run at 50°C for 2 min, 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. Cycle threshold (C_t) values were determined for each sample. Changes in the expression of target genes were calculated using the equation of $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t=(C_t^{target}-C_t^{GAPDH})_{sample}-(C_t^{target}-C_t^{GAPDH})_{control}$.

The statistical differences between the effects of the various titanium surfaces on the cultured cells were analyzed by one-way analysis of variance (ANOVA) for repeated measures followed by *t*-test. All statistical analyses were performed using SPSS (Statistics Software v 19.0; IBM, Armonk, NY, USA).

RESULTS

Representative images of attached HUVECs on different Ti surfaces after 24 h of incubation time are shown in Fig. 1. No notable differences in cell morphology were observed between cells cultured on different surfaces.

In the absence of EMD the cell number of HUVECs grown on TCP was significantly higher compared to PT and A surfaces (Fig. 2). EMD induced a significant decrease in the cell number of HUVECs grown on SLA surface. On the other surfaces, no significant effect of EMD on HUVECs number was observed.

In the absence of EMD, proliferation/viability in cells grown on tissue culture plastic was significantly higher compared to the cells grown on all Ti surfaces ($p<0.05$, Fig. 3). Within Ti surfaces, proliferation/viability of HUVECs grown on moderately rough SLA surface was significantly higher compared to cells grown on smoother PT and A surfaces. EMD inhibited cell proliferation/viability in cells grown on tissue culture plastic, A and SLA surfaces but not on PT surfaces.

The expression of ICAM-1 and E-selectin was significantly higher in HUVECs grown on smooth PT surface compared to TCP (Fig. 4). In contrast, cells grown on moderately rough SLA surface exhibited significantly lower expression of ICAM-1 and E-selectin compared to TCP. EMD induced a significant increase in the expression of ICAM-1 and a significant decrease

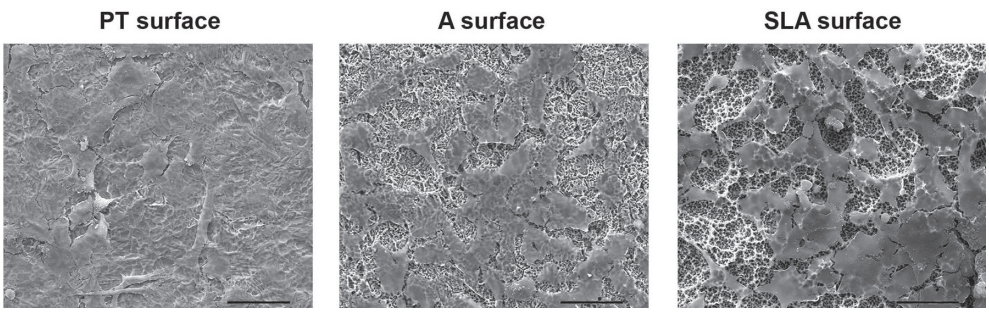


Fig. 1 Representative SEM images of PDLs after 24 h incubation.
HUVECs were seeded on PT, A, and SLA surfaces and cultured for 24 h and SEM images were taken. Horizontal bar corresponds to 50 μm.

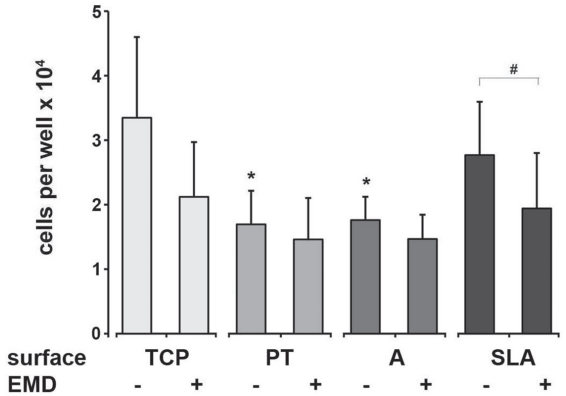


Fig. 2 Cell number of HUVECs grown on different surfaces in the presence or in the absence of EMD. HUVECs were cultured on different surfaces in the presence or in the absence of EMD (50 μg/mL) for 48 h and cell number was measured by manual counting. The data are presented as the mean±SD of three experiments. *Significant differences between groups of the same surface roughness cultured in the presense or absence of EMD ($p<0.05$).
* significantly different PT *vs*. TCP, A *vs*. TCP, SLA *vs*. TCP
# significantly different surface without EMD *vs*. surface with EMD

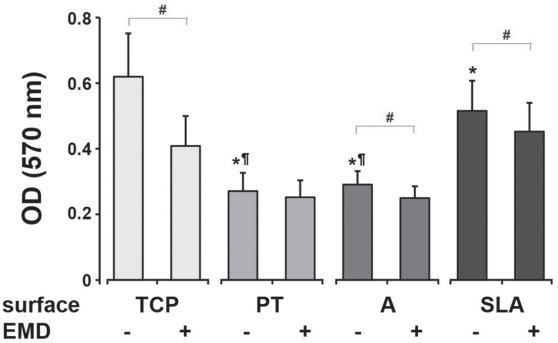


Fig. 3 Proliferation/viability of HUVEC grown on different surfaces in the presence or in the absence of EMD. HUVECs were cultured on different surfaces in the presence or in the absence of EMD (50 μg/mL) for 48 h and cell proliferation/viability was measured by MTT assays. The data are presented as the mean±SD of six experiments. *Significant differences between groups of the same surface roughness cultured in the presense or absence of EMD ($p<0.05$).
* significantly different PT *vs*. TCP, A *vs*. TCP, SLA *vs*. TCP
¶ significantly different PT *vs*. SLA, A *vs*. SLA
# significantly different surface without EMD *vs*. surface with EMD

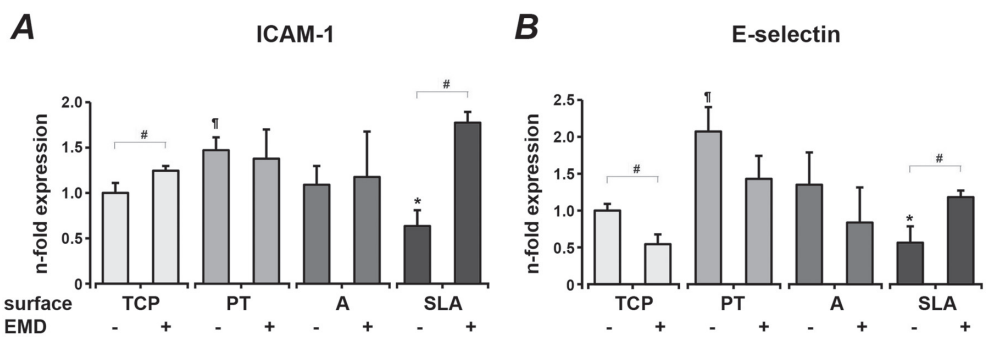


Fig. 4 Gene expression levels of adhesion molecules ICAM-1 and E-selectin in HUVECs grown on different surfaces in the presence or in the absence of EMD.
HUVECs were cultured on different surfaces in the presence or in the absence of EMD (50 μg/mL) for 48 h and the expression of adhesion molecules ICAM-1 (A) and E-selectin (B) was measured by qPCR. Y-axis represent n-fold expression in relation to HUVECs grown in tissue culture plastic. Data are presented as mean±s.e.m. of three independent experiments.
* significantly different SLA *vs*. TCP, SLA *vs*. PT, SLA *vs*. A
¶ significantly different PT *vs*. TCP
# significantly different surface without EMD *vs*. surface with EMD

in the expression of E-selectin in cells grown on TCP. The expression of both ICAM-1 and E-selectin was significantly increased by EMD (50 μg/mL) in cells grown on moderately rough SLA surface.

The expression of EPCR and vWF in cells grown on moderately rough SLA surface was significantly lower compared to TCP and smooth PT surface (Fig. 5). EMD in concentration of 50 μg induced a significant increase in the expression of both EPCR and vWF in cells grown on SLA surface. Besides, EMD induced a significant decrease in the expression of vWF in cells grown on TCP and PT surface.

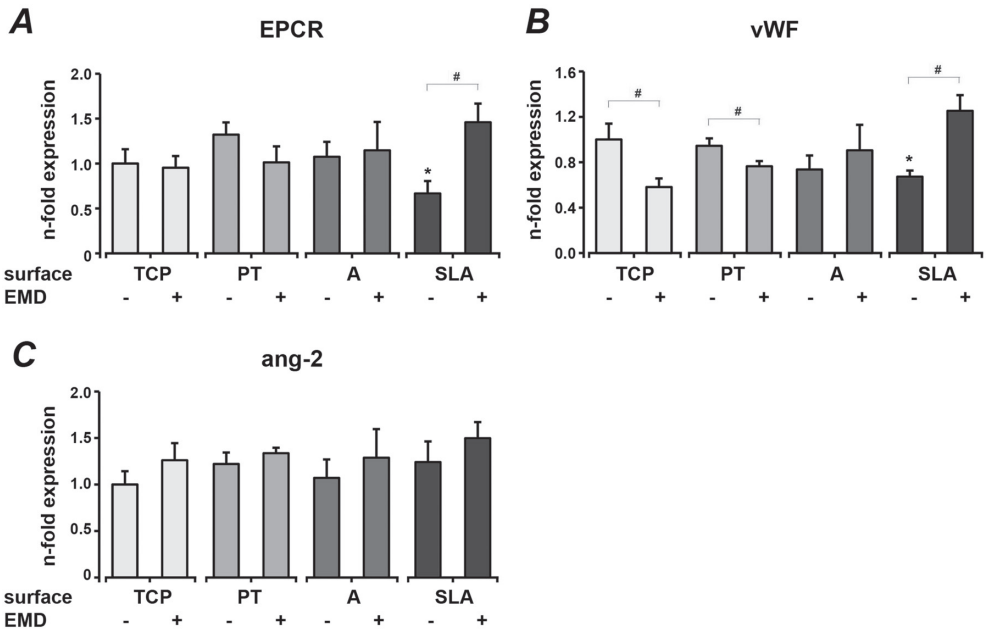


Fig. 5 Gene expression levels of EPCR, vWF, and ang-2 in HUVECs grown on different surfaces in the presence or in the absence of EMD.
HUVECs were cultured on different surfaces in the presence or in the absence of EMD (50 μg/mL) for 48 h and the expression of adhesion molecules EPCR (A), vWF (B), and ang-2 (C) was measured by qPCR. Y-axis represent n-fold expression in relation to HUVECs grown in tissue culture plastic. Data are presented as mean±s.e.m. of three independent experiments.
* significantly different SLA *vs.* TCP, SLA *vs*. PT
# significantly different surface without EMD *vs*. surface with EMD

DISCUSSION

Implementation of Ti implants with moderately rough surface topography resulted in substantially improved tissue integration of the implants[20], which is presumably associated with their positive effect on the proliferation and differentiation of osteoblasts[3,21,22]. One of the possible strategies for further development of the implant therapy could be enhancing the angiogenesis. After the onset of angiogenesis, EC escape from the blood vessel wall and proliferate to assemble the new vascular lumen. During this process, recruited ECs differentiate and start to express various angiogenic factors and secrete regulatory proteins. Angiogenesis might have a positive effect on the healing of both hard and soft tissues. Particularly, as recently reviewed, a close relation exists between angiogenesis, osteogenesis and osseointegration and sufficient angiogenesis is a pre-requisite for successful clinical outcome[23]. Impaired angiogenesis might result in delayed osseointegration and thus increase a gap period between primary and secondary implant stability. In addition, angiogenesis promote formation of peri-implant tissue, which prevent infection and implant failure due to peri-implantitis.

HUVECs proliferation on Ti surface was generally lower compared to tissue culture plastic. Within Ti surfaces, HUVECs proliferation was markedly higher on moderately rough SLA surface compared to smoother PT and A surfaces. This tendency was observed for both manual cell counting and MTT assay. Dependency of HUVECs proliferation on Ti surface roughness observed in the present study is similar to that observed in our previous works[9,10]. HUVECs proliferation was generally inhibited by EMD. Within Ti surfaces, the inhibition of cell proliferation by EMD was most pronounced on moderately rough SLA surface. This observation might be due to the fact that rougher surface has larger area for protein adsorption and might adsorb more EMD proteins.

Adhesion molecules ICAM-1 and E-selectin in endothelial cells mediate the adhesion of inflammatory cells to the endothelium and their migration to wound sites[24,25]. EPCR facilitates the interaction of protein C with the thrombin-thrombomodulin complex and accelerates thrombin mediated activation of protein C[26] and vWF is involved in the platelet adhesion[27,28]. In the present study we observed that the expression of ICAM-1, E-selectin, EPCR and vWF on moderately rough Ti surface is significantly lower compared to TCP and smoother Ti surfaces, which is similar to previous

observations[9,10]. The expression of all these angiogenesis-related factors was significantly enhanced by EMD in HUVECs grown on SLA surface, but not on the smoother Ti surfaces. This observation suggests that interaction of EMD with moderately rough Ti surface could be an important factor influencing its biological activity.

To our knowledge, this is the first study concerning HUVEC behavior on different Ti surfaces in the presence of EMD. Some previous studies showed that EMD stimulates differentiation of osteoblasts grown on moderately rough Ti surfaces[17,19]. One of these studies show that EMD promotes the proliferation and differentiation of osteoblasts an both PT and SLA surfaces, which suggest that the effect of EMD on osteoblasts does not depend on the Ti surface topography[29]. In contrast to this finding, we have observed that surface roughness is an important factor influencing the response of endothelial cells to EMD. This discrepancy might be explained by different dependencies on the functional activity of osteoblasts and endothelial cells on surface roughness. It is well known that differentiation of osteoblasts is promoted by moderately rough surfaces[3,30], whereas endothelial cells prefer smoother surfaces[9].

The exact mechanisms underlying the promoting effect of EMD on endothelial cells and angiogenesis remain unclear. EMD is composed mainly of amelogenin and amelogenin transcripts resulting of gene alternative splicing[31-35]. A recent study reported that different EMD components may possess different biological activity[31], however their exact bioactive specificities in respect to different tissues and cells need to be investigated. A few studies also suspected the presence of transforming growth factor β1 in EMD[36]. In addition, EMD might contain other not yet detected bioactive components. Some recent studies suggest that tyrosine rich amelogenin peptide, which is N-terminal peptide of amelogenin, has strong angiogenic activity[13,37,38].

Moderately rough surfaces show excellent outcome regarding osseointegration, but seem to be not optimal for angiogenesis[9,10]. In our study we tried to compensate the negative effect of moderately rough Ti surface on ECs using bioactive substance such as EMD. We used EMD at concentration of 50 μg/mL, because our previous study shows that this concentration has the most pronounced effect on ECs[16]. We assume that in our study the effect of EMD on ECs is due to direct effect on the cells rather than on their interaction with Ti surface due to EMD adsorption. According to our study design, cells were first seeded on the surface at the cell culture medium supplemented with 20% FCS and let them attach for 24 h. Within this time, FCS proteins are adsorbed by Ti surface, which make possible ECs attachment and growth on these surfaces. We added EMD only after 24 h and it is hardly to imagine that EMD protein might substitute the proteins adsorbed by Ti surface. However, the activation of intrinsic signaling pathways of ECs by EMD might influence their interaction with Ti surfaces. Stimulation of the expression of angiogenesis associated proteins in HUVECs was most prominent in cells grown on rougher Ti surfaces. This finding suggests that application of EMD might counterweigh the inhibition of endothelial cells function by moderately rough surfaces, which are widely used for the implantation. However, interpolation of the data obtained in the present *in vitro* study to *in vivo* application should be done carefully, since application of EMD *in vivo* results in complex response, which includes various cell types.

CONCLUSION

Summarizing, our data suggest that EMD stimulates the expression of angiogenesis associated proteins in HUVECs grown on rougher Ti surfaces. Further research should be required to evaluate the effect of EMD on angiogenesis around Ti surfaces by the animal experiment and clinical trials.

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