Clin Oral Invest (2016) 20:685–695
DOI 10.1007/s00784-015-1558-5

ORIGINAL ARTICLE

Enamel matrix derivative improves gingival fibroblast cell behavior cultured on titanium surfaces

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Received: 2 March 2015 / Accepted: 31 July 2015 / Published online: 14 August 2015
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Abstract
Objective Although an extensive amount of research has demonstrated the positive effects of an enamel matrix derivative (EMD) on soft tissue wound healing around intrabony defects, little information is available describing its effect on peri-implant soft tissues, an area that has recently gained tremendous awareness due to the increasing prevalence of peri-implantitis. The aim of the present study was to assess the role of EMD when gingival fibroblasts were cultured on titanium surface with different surface topographies.
Methods Human primary gingival fibroblasts were cultured on pickled (PT) and sand-blasted with large grit followed by acid etching (SLA) surfaces and assessed for cell adhesion at 2, 4, and 8 h, cell morphology at 2, 4, 8, and 24 h as well as cell proliferation at 1, 3, and 5 days post-seeding. Furthermore, genes encoding collagen 1a1, vascular endothelial growth factor-A (VEGF-A), and fibronectin were assessed by real-time PCR. Human gingival fibroblasts were also quantified for their ability to synthesize a collagen matrix on the various titanium surfaces with and without EMD by immunofluorescence staining.
Results The results from the present study demonstrate that EMD significantly increased cell spreading at 2, 4, 8, and 24 h on PT surfaces and 4, 8, and 24 h on SLA surfaces. Furthermore, proliferation at 5 days on PT surfaces and 3 and 5 days on SLA surfaces was also increased for groups containing EMD. Real-time PCR results demonstrated that the culture of gingival fibroblasts with EMD significantly increased extracellular matrix synthesis of collagen 1 as well as improved mRNA levels of VEGF-A and fibronectin. Collagen1 immuno-fluorescent staining revealed a significantly higher area of staining for cells seeded on PT + EMD at 7 and 14 days and 14 days for SLA + EMD when compared to control samples.
Conclusion The results from the present study favor the use of EMD for colonization of gingival fibroblasts on titanium surfaces by increasing cell growth, spreading, and synthesis of an extracellular matrix. The improvements were primarily irrespective of surface topography. Future animal and human studies are necessary to fully characterize the beneficial effects of incorporating EMD during soft tissue regeneration of implant protocols.
Clinical relevance The use of EMD may speed up the quality of soft tissue integration around dental implants by facilitating gingival cell attachment, proliferation, and matrix synthesis of collagen 1.

Keywords Gingival fibroblasts · Soft tissue healing · Titanium implants · Enamel matrix derivative · Emdogain

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Introduction

Enamel matrix derivative (EMD) has been used for a variety of clinical applications for the regeneration of lost periodontal tissues including cementum, periodontal ligament, and

alveolar bone [1]. The major components of EMD, amelogenins, are a group of hydrophobic proteins that account for approximately 95% of the total protein content [2]. These proteins self-assemble into supramolecular aggregates that form an insoluble extracellular matrix that have as function to control the ultrastructural organization of developing enamel crystallites [2]. Other proteins that have been reported in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin, and various proteinases [3, 4].

The rationale for using EMD is the observation that amelogenins, which until then were considered an enamel-specific protein, were found deposited onto the surface of developing tooth roots prior to cementum formation [5–10]. It was later observed that these proteins largely contribute to the differentiation of periodontal tissues into new cementum formation, and thereafter a series of studies has demonstrated the role of EMD in osteoblast differentiation and periodontal ligament formation [11–14]. The commercially available extract of EMD (Emdogain®, Straumann AG, Basel, Switzerland) has been demonstrated to successfully stimulate the regeneration of periodontal tissues and increase clinical attachment levels when employed on denuded root [11, 12, 15].

Recently, the effects of EMD on soft tissue wound healing and inflammatory resolution were investigated in a review article [16]. It was found that EMD has the ability to significantly decrease interleukin-1b and RANKL expression, increase prostaglandin E2 and OPG expression, increase proliferation and migration of T lymphocytes, induce monocyte differentiation, increase bacterial and tissue debris clearance, as well as increase fibroplasias and angiogenesis by inducing endothelial cell proliferation, migration, and capillary-like sprout formation. The outcomes from this review article indicate that EMD is able to affect substantially the inflammatory and healing responses, and further clinical reports also point to the fact that EMD is able to increase wound healing in periodontal tissues [17–21]. This is further exemplified by the fact that EMD is also successfully packaged and sold under the trademark name Xelma® (Mölnlycke Health Care, Gothenburg, Sweden) for the treatment of hard-to-heal skin ulcers such as venous leg ulcers, diabetic foot ulcers, and pressure ulcers, by functioning as a temporary matrix that improves cell adhesion and subsequent tissue/wound healing [22–30].

One area of research that has gained tremendous awareness in recent years is that of peri-implantitis [31–37]. With a continuously rising number of patients now affected by peri-implantitis, the need for a better understanding of the interactions that take place during its progression becomes vital for its future resolution. Since the majority of the initial research in the field of implant dentistry focused on hard tissue osseointegration of titanium implants [38–41], much less focus has addressed the soft tissues surrounding the implant collar. The gingival/epithelial seal around the implant collar is thought to prevent bacterial infiltration and, a better attachment of gingival cells around the implant collar may inhibit disease progression of peri-implantitis. Since EMD has been shown to increase soft tissue wound healing, increase extracellular matrix synthesis, and speed up inflammatory resolution, the aim of the present study was to investigate the relationship between gingival cells on titanium surfaces with and without EMD. Therefore, human primary gingival fibroblasts were cultured on pickled (PT) and sand-blasted with large grit followed by acid etching (SLA) surfaces and assessed for cell adhesion, cell morphology, as well as cell proliferation at various time intervals. Furthermore, genes encoding extracellular matrix formation including collagen 1, vascular endothelial growth factor-A (VEGF-A), and fibronectin were assessed by real-ime PCR. Collagen 1 staining following 1 and 2 weeks of cell culture was assessed by immunofluorescence to assess the quantity and morphology of the extracellular matrix on the various implant surfaces with and without EMD.

Materials and methods

Preparation of PT and SLA titanium discs

Grade 2 unalloyed Ti sheets were punched into 5-mm-diameter samples. Smooth pickled Ti (PT) surfaces were prepared using dilute nitric acid to clean the surfaces, followed by washing in reverse-osmosis-purified water. Roughened SLA topography surfaces were prepared by blasting the Ti with corundum particles, followed by etching with HCl/H_2SO_4. The surface topographies of PT and SLA surfaces can be visualized in Fig. 1.

EMD preparation

EMD was prepared according to Institut Straumann AG standard as previously described [42–44]. Briefly, 0.7 ml (one vial) of 30 mg/ml EMD was dissolved in 1.4 ml of 4C sterile 0.1% acetic acid to make a stock solution of 10 mg/ml. During experimental seeding, stock EMD was diluted with 1:100 in α-MEM (Hyclone, Thermo Fisher Scientific Inc, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Hyclone, Thermo Fisher Scientific Inc). Samples containing EMD were then seeded in plates together with human gingival fibroblasts, while control samples without EMD were seeded containing medium alone which contains 10% FBS.

Isolation of human gingival fibroblasts

Gingival tissues were harvested from healthy donors undergoing third molar extraction. Ethical approval and consent was obtained from all volunteers. Collected tissues were washed three times with phosphate-buffered saline (PBS;

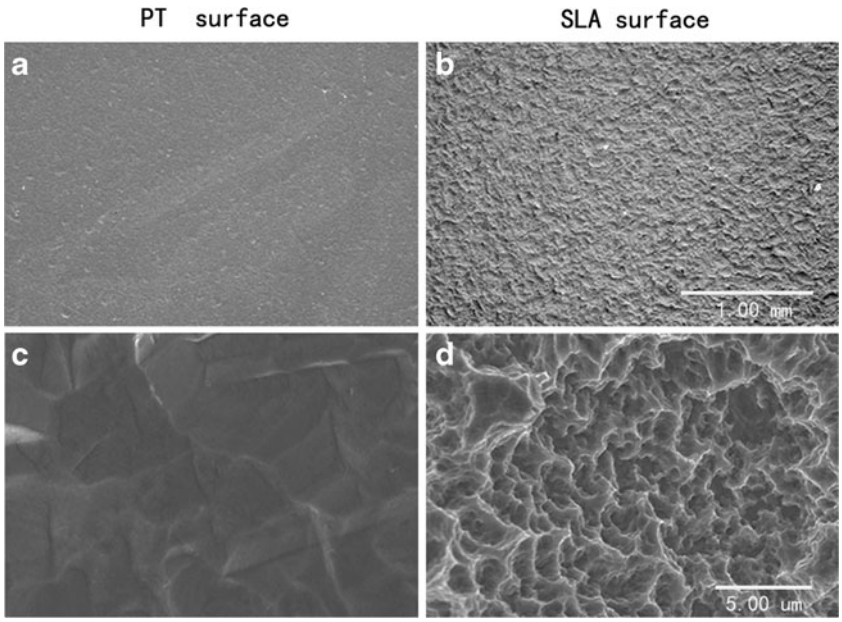

Fig. 1 Scanning electron microscopy of PT and SLA surfaces at ×40 (**a** and **b**) and ×6000 (**c** and **d**) magnification

150 mM NaCl, 20 mM sodium phosphate, pH 7.2) supplemented with antibiotics (100 U/ml penicillin,100 mg/ml streptomycin) and cut into small pieces with sterilized surgical scissors.

The gingival tissue pieces were then transferred into T25 tissue culture flasks containing minimal α-MEM and were allowed to adhere for 2 h. Then, 3 ml of α-MEM containing 20% fetal bovine serum and antibiotics (100 U/ml medium of penicillin, 100 mg/ml streptomycin, HyClone, Thermo Fisher Scientific Inc) was added. Cultures were maintained in an incubator at 37°C in an air atmosphere with 95% humidity and 5% CO_2. After 1 week, when cells reached confluency, cells were trypsinized, and cultures were expanded in α-MEM containing 10% fetal bovine serum. Human gingival fibroblasts used for experimental analysis were chosen from passages 2–4.

Cell adhesion and proliferation assays

Cells were seeded onto titanium discs in 24-well plates at a density of 1.5×10^4 cells per Ti structure and cultured for 2, 4, and 8 h for the adhesion assay. Cell number was measured by staining cells with 4′,6-diamidino-2-phenylindole (DAPI) as previously described [45]. At each time point, the Ti structures were washed with PBS to remove non-attached cells and fixed in 4% formaldehyde for 10 min, followed by staining with DAPI. Images were captured on an Olympus DP71 fluorescence microscope (Olympus Co, Japan). Ten fields of view were captured per sample, and nuclei were counted using Image J software as previously described. For the cell proliferation assays, HGFs were seeded on titanium discs in 96-well plates at a density of 2000 cells per well with culture medium with versus without EMD. At time points 1, 3, and 5 days, cell number of HGFs was determined by the Cell Counting Kit-8 (Dojindo, Japan) and measured by a microplate reader scanning at 450 nm (PowerWave XS2, BioTek, Winooski, VT, USA) as previously described [46]. Samples were performed in triplicate with three independent experiments performed.

Cell morphology

Human gingival fibroblasts were plated at a density of 10,000 cells on PT and SLA surfaces either with versus without EMD in a 24-well plate. At 2, 4, 8, and 24 h, cells were fixed using 4% formaldehyde followed by rinsing with PBS for 5 min. Then, cells were stained with 5 ug/ml phalloidin-FITC (Sigma–Aldrich) for 1 h in dark conditions at 37°C as previously described [44]. Finally, a drop of DAPI was added for 3 min to visualize the cell nuclei. Images were captured from each surface on an Olympus DP71 fluorescence microscope (Olympus Co, Japan) for samples with and without EMD and compared for morphological differences. The planar area of the cells was measured using Image J software. A minimum of 50 cells from each treatment group was counted and analyzed for statistical variance between samples as previously described [44].

ECM-related gene expression

For real-time PCR experiments, 10,000 cells were seeded onto PT and SLA surfaces with and without EMD. After 5 days of culture, total RNA was isolated from HGF cells using

AxyPrep™ Multisource Total RNA Miniprep Kit (AXYGEN, Union City, CA, USA) according to the manufacturer's protocol. The RNA concentration was determined by a NanoDrop 2000 UV–vis spectrophotometer as previously described [47]. A total of 1 ug RNA solution was immediately reverse-transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), and the final volume is 100 ul. The sequences of primers for collagen type I alpha1 (COL1A1), VEGF-A, fibronectin (FN1), and GAPDH genes of human are listed in Table 1. Real-time RT-PCR was performed using 25 ul final reaction volume of QuantiFast™ SYBR Green PCR Kit (QIAGEN, Venlo, Holland). Two microliters of total RNA was used per sample well. Quantitative real-time RT-PCR was performed with a CFX Connect™ Real-Time PCR Detection System. The delta-delta Ct method was used to calculate gene expression levels relative to house-keeping gene GAPDH and normalized to control cells (blank well without Ti structure). Each sample contained pooled mRNA collected from ten Ti surfaces, and all samples were log-transformed. All samples were performed in triplicate with three independent experiments performed.

Collagen type I staining

HGFs were plated at a density of 5000 cells per structure in a 24-well plate. The culture medium was changed every 3 days. At 1 and 2 weeks, Ti structures were washed with PBS and fixed with 4% formaldehyde for 10 min at room temperature (RT). Cells were then permeabilized with 0.5% Triton X-100 (Merck, Germany) in PBS for 3 min at room temperature. Subsequently, cells were washed and incubated for 1 h at 37°C with polyclonal rabbit to collagen type I (1:100) (Boster Biological Technology Ltd, Wuhan, China) diluted 1:100 in PBS containing 2% bovine serum albumin (BSA, Roche). After washing with PBS, cells were incubated for 1 h at 37°C with FITC-conjugated-goat-anti-rabbit (Invitrogen) (1:500) diluted in PBS containing 2% BSA. The cells were washed again and incubated with DAPI for 3 min. Images were captured from each surface on an Olympus DP71 fluorescence microscope (Olympus Co, Japan). The optical density (OD) of the fluorescent collagen staining was quantified from three independent experiments using Image J software.

Table 1 Sequences of primers utilized for VEGF-A, FN1, COL1A1, and GAPDH

Genes	Primer sequence
VEGF-A-F	GAGCCTTGCCTTGCTGCTCTAC
VEGF-A-R	CACCAGGGTCTCGATTGGATG
FN1-F	ACCTACGGATGACTCGTGCTTTGA
FN1-R	CAAAGCCTAAGCACTGGCACAACA
COL1A1-F	TCTAGACATGTTCAGCTTTGTGGAC
COL1A1-R	TCTGTACGCAGGTGATTGGTG
GAPDH F	GCACCGTCAAGGCTGAGAAC
GAPDH R	TGGTGAAGACGCCAGTGGA

Statistical analysis

Statistical analyses was analyzed by one-way ANOVA with Bonferroni test, using Graphpad Software v. 4 (Graphpad Software, La Jolla, CA, USA), and statistical significance was considered at $p < 0.05$. All data are expressed as the mean±SE.

Results

Implant surface topography

SEM images of PT and SLA surfaces demonstrated markedly different surface topographies (Fig. 1). At a magnification of ×40, PT surfaces appear relatively smooth with only slightly appearing valleys observed at ×6000 magnification (Fig. 1c). In contrast, SLA surfaces appeared roughened at ×40 magnification with many micro and nanotopographies visualized at ×6000 magnification (Fig. 1d).

Human gingival fibroblast attachment

HGFs attached similarly on both PT and SLA surfaces with no significant difference being observed with or without EMD at all time points (Fig. 2). At 2 h post-seeding, it was observed that approximately 20–25 cells per field of view were attached to all surfaces irrespective of EMD coating with no significant difference observed between groups (Fig. 2). By 4 h, cell numbers had increased to 40–45 cells per group irrespective of surface topography or the presence of EMD (Fig. 2). This was consistent at 8 h where no significant difference could be observed between all treatment groups (Fig. 2). Analysis of cell shape by immunofluorescence staining demonstrated that cells seeded on PT surfaces alone attached at 2 h; however, their appearance was still round with very little cell spreading observed (Fig. 3). In contrast, when human gingival fibroblasts were seeded with EMD, cells began to spread on PT surfaces (Fig. 3). A similar observation was observed throughout the time course of these experiments with the highest cell spreading visually observed for cells seeded on PT surfaces in combination with EMD (Fig. 3). The average planar area of cells seeded on the different surfaces with/without EMD was then quantified for spreading area (Fig. 4). It was first observed at 8 and 24 h that cells seeded on SLA alone had significantly less spreading when compared to PT alone ($p<0.05$; Fig. 4). Interestingly, PT samples cultured with

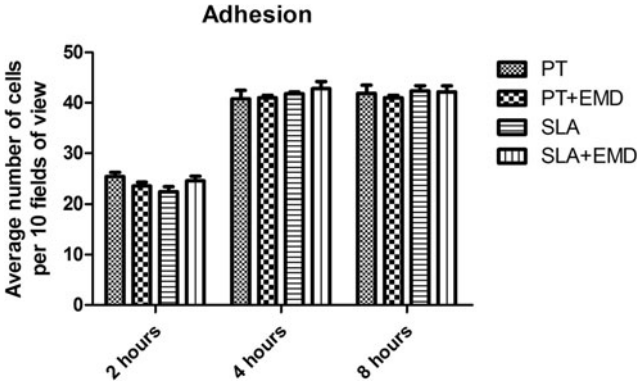

Fig. 2 Effects of surface topography and EMD on human gingival fibroblast cell adhesion. Average cell number of cells seeded on PT control, PT EMD, SLA control, and SLA+EMD at 2, 4, and 8 h post-seeding. No significant differences were observed for human gingival fibroblast attachment levels between EMD-treated surfaces versus control surfaces at all time points. Data are means ± SE

EMD demonstrated significantly higher cell spreading when compared to PT alone samples at all time points, and cells seeded on SLA surfaces with EMD additionally demonstrated significantly higher values at 4, 8, and 24 h when compared to SLA alone (Fig. 4).

Human gingival fibroblast proliferation

The analysis of cell proliferation demonstrated significant changes in cell number that were influenced by both surface topography as well as EMD (Fig. 5). At 1 day post-seeding, an equal number of cells was observed on all surfaces with an equal number of cells irrespective of EMD or surface topography (Fig. 5). By 3 days, however, it was observed that cells seeded on SLA demonstrated significantly lower cell numbers when compared to PT surfaces (Fig. 5). When EMD was added to culture media, HGFs seeded on SLA surfaces demonstrated significantly higher cell numbers than HGFs seeded on control SLA surfaces without EMD at 3 days (Fig. 5). No significant difference was observed between PT+EMD and control PT surfaces at 3 days post-seeding (Fig. 5). By 5 days, it was again observed that the control SLA surfaces demonstrated significantly lower cell numbers when compared to PT surfaces (Fig. 5). Furthermore, cells seeded with EMD on both PT and SLA surfaces demonstrated significantly higher cell numbers at 5 days when compared to their respective controls (Fig. 5).

Human gingival fibroblast expression of FN1, VEGF-A, and COL1A1

Gingival fibroblasts were investigated for mRNA levels for genes encoding FN1, VEGF-A, and COL1A1 as assessed by real-time PCR at 5 days post-seeding (Fig. 6). COL1A1 demonstrated significantly higher levels of mRNA on both PT and SLA surfaces when HGFs were seeded with EMD at 5 days post-seeding (Fig. 6a). No differences could be observed between control PT and SLA surfaces. Analysis of mRNA levels of cell adhesion molecule FN1 demonstrated that cells seeded in the presence of EMD also demonstrated elevated levels of FN1 when compared to their respective control surfaces (Fig. 6b). It was also observed that FN1 expression was higher on SLA surfaces when compared to PT surface irrespective of EMD (Fig. 6b). This trend was also observed for the expression of VEGF-A (Fig. 6c). HGFs seeded with EMD

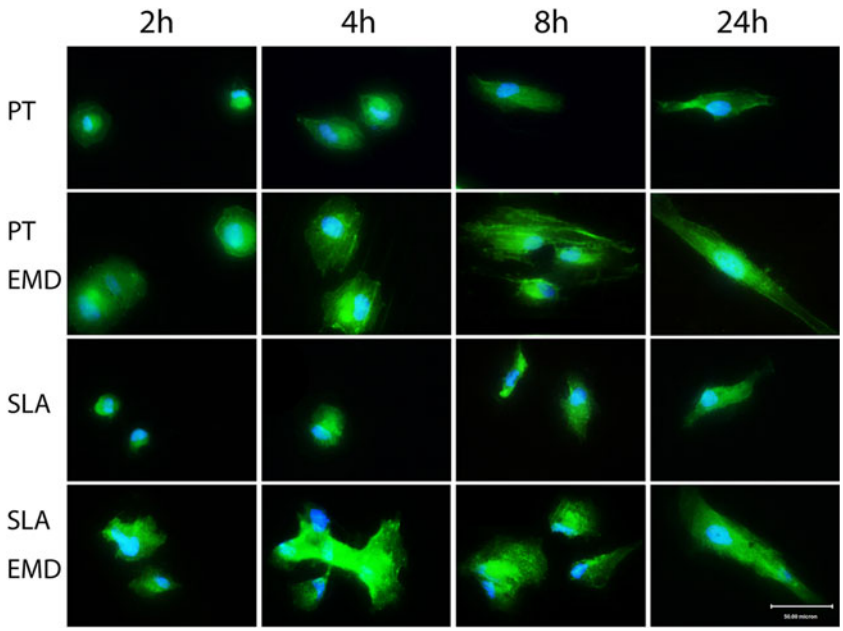

Fig. 3 EMD promotes initial cell spreading of human gingival fibroblasts (HGFs) on both PT and SLA titanium surfaces at time points 2, 4, 8. and 24 h. Cells were stained for F-actin (*green*) and nuclei (*blue*). HGFs attached to PT surfaces were well spread and circular in shape by 2 h and continued to spread throughout the experiment. On SLA surfaces, HGFs formed more elongated shapes with microspikes observed due to the confinements of the roughened surface topography

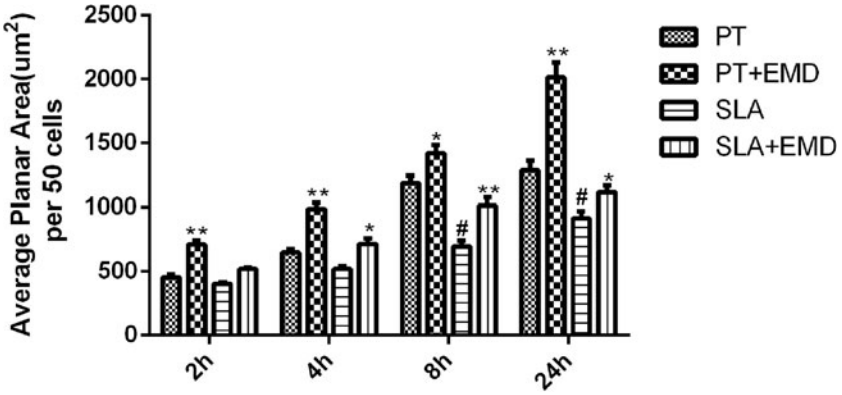

Fig. 4 Average planar area of cells attached to PT and SLA surface with and without EMD at 2, 4, 8 and 24 h post-seeding. EMD significantly increased cell spreading on PT surfaces at all time points and on SLA surfaces at 4, 8, and 24 h. It was also noted that surface cell spreading was significantly greater on PT surfaces at 8 and 24 hours when compared to SLA surfaces. Data are means ± SE. (*Single asterisk* and *double asterisks* denote significant differences between EMD-treated surface and respective control surfaces, $p<0.05$ and $p<0.01$, respectively. *Number sign* denotes differences between PT and SLA surfaces ($p<0.05$). Data are means ± SE)

demonstrated significantly higher levels of VEGF-A when compared to their respective controls (Fig. 6c). Furthermore, cells seeded on control SLA surfaces also demonstrated significantly higher levels when compared to control PT surfaces (Fig. 6c).

Collagen type I staining

Analysis of collagen type 1 expression was investigated in order to visualize and quantify the extracellular matrix deposition of human gingival fibroblasts over time on both PT and SLA surfaces (Fig. 7). It was observed that at 7 days post-seeding, most of the collagen expression was localized intracellularly with little deposition observed on PT and SLA surfaces without EMD (Fig. 7). In cell culture with EMD, immunofluorescence of collagen 1 was gradually observed outside the cells with an extracellular matrix deposited within the cell's periphery (Fig. 7). By 2 weeks, a continuous layer of extracellular matrix was observed on both surfaces receiving EMD (Fig. 7). It was observed also that a more uniform layer of collagen 1 staining was observed on PT surfaces when compared to SLA, which is likely due to surface topography (Fig. 7). Interestingly, the control PT and SLA samples began to secrete collagen outside the cell; however, non-uniformity was observed with very little collagen synthesis observed between cells (Fig. 7). Quantification of staining area revealed a significantly higher amount of staining area observed on PT surfaces coated with EMD when compared to PT alone at both 7 and 14 days (Fig. 8). A statistically significant difference was observed for SLA + EMD when compared to SLA alone at 2 weeks only (Fig. 8). No significant difference could be observed between control PT and control SLA surfaces at either time point (Fig. 8).

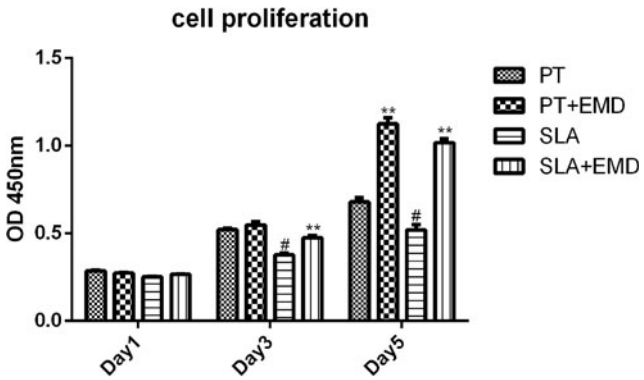

Fig. 5 Effects of surface topography and EMD on human gingival fibroblast cell proliferation for PT control, PT+EMD, SLA control, and SLA+EMD samples. EMD significantly increased cell numbers on PT surfaces at 5 days and on SLA surfaces at both 3 and 5 days post-seeding. Data are means ± SE. (*Double asterisks* denote significant differences between EMD-treated surface and respective control surfaces, $p<0.01$. *Number sign* denotes differences between PT and SLA surfaces ($p<0.05$). Data are means ± SE)

Discussion

EMD is a widely used biologic agent capable of enhancing periodontal wound healing/regeneration [1]. A systematic review on the in vitro roles of EMD has been well documented in both PDL cells, cementoblasts and osteoblasts [14]. EMD has a significant influence on cell adhesion, cell proliferation, and cell differentiation of many cell types by mediating cell attachment, spreading, proliferation, and survival as well as expression of transcription factors, growth factors, cytokines, extracellular matrix constituents, inter-cellular communication molecules, and other molecules involved in the regulation of bone remodeling [14, 45]. Furthermore, there have been numerous reports that also indicate that EMD is able to significantly upregulate osteoblast proliferation or differentiation of hard tissues on titanium surfaces in vitro and increase the speed and quality of new bone formation in vivo [44, 48–51].

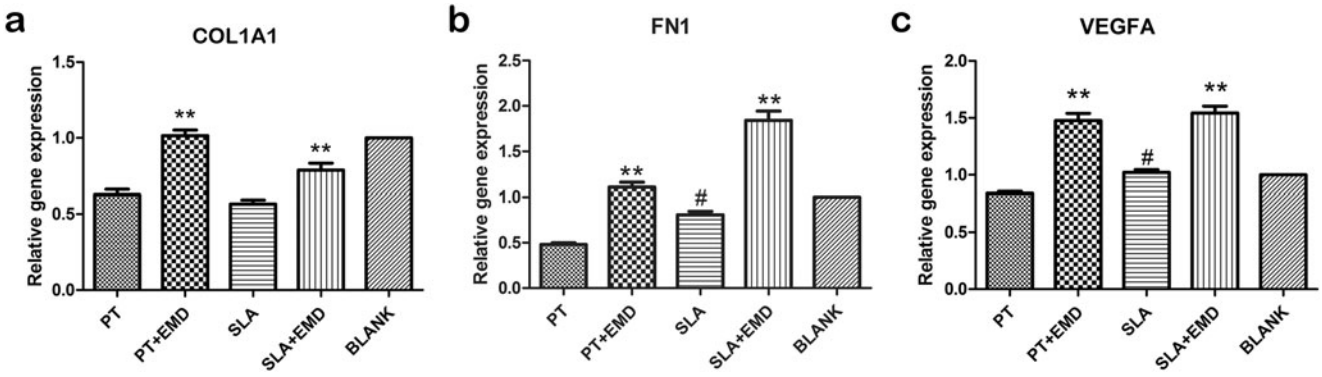

Fig. 6 Real-time PCR of genes encoding **a** collagen 1 (*COL1A1*), **b** fibronectin-1 (*FN1*), and **c** vascular endothelial growth factor-A (*VEGFA*) for human gingival fibroblasts seeded on PT and SLA surfaces with and without EMD 5 days post-seeding. It was observed that EMD was able to significantly upregulate COL1A1, FN1, and VEGF-A irrespective of surface topography. (*Double asterisks* denote significant differences between EMD-treated surface and respective control surfaces, $p<0.01$. *Number sign* denotes differences between PT and SLA surfaces ($p<0.05$). Data are means ± SE)

The aim of the present study was to investigate the potential role of EMD on soft tissue healing around titanium implants with different surface topographies. As such, human primary gingival fibroblasts were seeded on both PT and SLA surfaces and analyzed for cell behavior. It was found that cells attached equally to titanium surfaces irrespective of EMD; however, EMD had a more prominent influence on cell spreading of HGFs. It has previously been demonstrated that EMD, which is typically packaged in an acidic medium with pH ranging from 4 to 5, will rapidly precipitate to the surface of either titanium surfaces [44], bone grafting materials [43, 52, 53], and denuded root surfaces [54] once the pH reaches physiological pH at approximately 7. Once adsorption has taken place to the surface of the various materials, the amelogenin aggregates serve as a future protein layer for cell attachment able to initiate cell spreading [55].

It was also noted in the present study that EMD had a significant influence on cell proliferation of gingival fibroblasts seeded on either surface at 5 days. Many numerous reports have also demonstrated that EMD is able to increase gingival cell proliferation on cell culture plastic, with one recent report demonstrating significant increases on zirconia surfaces [56–61]. It was previously found that EMD induces proliferation of human gingival fibroblasts via activation of extracellular regulated kinase (ERK) [61] as well as increased tyrosine phosphorylation of epidermal growth factor receptor [60]. Although we have not investigated in detail the cellular pathways responsible for the significant increases in cell proliferation of gingival fibroblasts cultured on titanium surfaces, it is likely that the proliferative effects of EMD are via similar pathways [60–62]. Importantly also, it was found that the effects were noticed on implant surfaces irrespective of surface topography. There has been a great deal of research performed over the years that has focused on implant surface topography on both osteoblast and fibroblast cell behavior [63–66]. Consistent with previous reports from the literature,

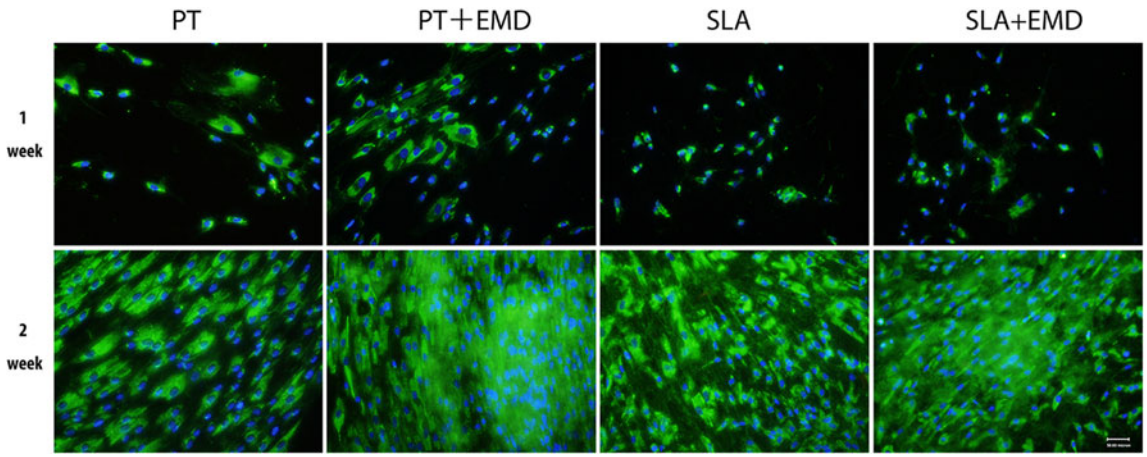

Fig. 7 Immunofluorescence staining of collagen type 1 for human gingival fibroblasts seeded on PT and SLA surfaces with and without EMD. It was observed that EMD was able to promote extracellular matrix deposition and that fluent collagen staining was observed 2 weeks post-seeding on PT + EMD and SLA + EMD surfaces

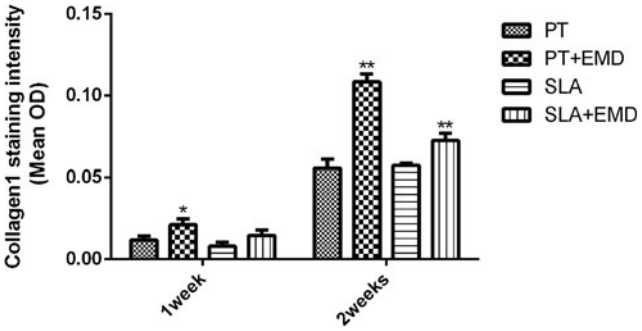

Fig. 8 Quantification of immunofluorescence staining of collagen type 1 of gingival fibroblasts seeded on PT and SLA surface with and without EMD at 1 and 2 weeks post-seeding. It was observed that EMD significantly increased collagen 1 staining on PT surfaces at 1 and 2 weeks and at 2 weeks for SLA surfaces. No significant differences could be observed between PT and SLA surfaces. Data are means ± SE. (*Single asterisk* and *double asterisks* denote significant difference between EMD treated surface and respective control surfaces, $p<0.05$ and $p<0.01$, respectively)

it was found in this study that surfaces with rougher topographies demonstrate reduced cell proliferation and an increase in extracellular matrix synthesis [62].

We then analyzed the mRNA levels of fibronectin, VEGF, and collagen 1 via real-time PCR (Fig. 6). It was first noted that EMD significantly increased the levels of fibronectin when compared to controls on both PT and SLA surfaces (Fig. 6b). Fibronectin is a glycoprotein that binds to extracellular matrix components such as collagen, fibrin, and heparin and aids in cell attachment and future extracellular matrix synthesis [67, 68]. It was also observed that SLA surfaces were able to stimulate higher levels of fibronectin mRNA levels when compared to PT surfaces (Fig. 6b). Previously, it has been shown that substratum surface topography alters the cell shape of gingival fibroblasts and modulates fibronectin at the transcriptional and post-transcriptional levels [64]. Thus, it may be concluded that not only did surface topography induce additional expression of FN consistent with the literature [64] but also EMD seems to further enhance FN mRNA levels on both PT and SLA surfaces.

We then characterized the effects of surface topography on gingival cell mRNA expression of VEGF-A, a growth factor responsible for angiogenesis. It was first found that surface topography with a roughened surface enabled higher mRNA levels of VEGF-A (Fig. 6c). Furthermore, EMD markedly improved VEGF expression on both PT and SLA surfaces, and this finding was similar to previous reports from the literature demonstrating the effects of EMD on VEGF production on tissue culture plastic [69, 70]. Aspriello et al. assessed if topical EMD application in an instrumented periodontal pocket could affect angiogenesis at the gingival level in 56 defects in 28 humans [69]. After 48 h, gingival biopsies were collected for histologic and immunohistochemical analysis for VEGF and CD34 (for microvessel density [MVD] count) antibodies. It was reported that EMD induces proliferation, viability, and angiogenesis of human microvascular cells from human biopsies [69]. Furthermore, Sakado et al. investigated the effect and the mechanism of action of EMD on VEGF production by human gingival fibroblasts [70]. Their results suggest that EMD stimulates VEGF production partially via TGF-β1 and fibroblast growth factor-2 in human gingival fibroblasts and that EMD-induced VEGF production is regulated by ERK, p38 MAPK, and PI3K/Akt pathways [70].

Interestingly, in the present study, all mRNA levels were normalized to control tissue culture plastic. For cells seeded on PT and SLA surfaces without EMD, it was found that gingival fibroblasts express higher mRNA levels of collagen and FN on tissue culture plastic when compared to implant surfaces of varying topographies. In light of these findings, it is likely that since tissue culture plastic is already pre-coated with adhesion molecules known to facilitate fibroblast attachment and growth, HGFs that were seeded on these surfaces are likely to immediately begin to synthesize molecules such as FN and COL1A1 quite rapidly. In contrast, implant surfaces that are completely devoid of any surface proteins may be retarded due to the fact that cells need to first lay extracellular matrix protein prior to attachment. Thus, cells seeded on control PT and SLA may be slightly slowed down in their secretion of COL1A1 and FN synthesis as they must initially produce their own extracellular matrix proteins for cell attachment (Fig. 6). Interestingly, the addition of EMD improved expression of both these genes when compared to tissue culture plastic on both surfaces (Fig. 6). Since EMD contains cell adhesion molecules such as amelogenin known to influence cell attachment, it is likely that additional bioactive molecules found in EMD which are known adhesion molecules are able to speed up the synthesis of COL1A1 and FN on various titanium surfaces. This hypothesis however requires further investigation.

The analysis of collagen 1 immunofluorescence staining was then performed to investigate the ability for gingival cells to secrete and synthesize a functional extra cellular matrix. It was found that EMD upregulared collagen 1 mRNA levels of gingival fibroblasts seeded on both PT and SLA surfaces (Fig. 7). More surprisingly was the pattern of ECM deposition on the various surfaces following exposure to EMD for a 2-week period. In all four groups, collagen was observed stained around the cells (Fig. 7). On the surfaces without EMD, however, there was very little staining interconnected between cells. The gingival fibroblasts seeded with EMD demonstrated more dense staining, and the appearance of a continuous ECM was apparent, especially on PT surfaces where collagen fibrils seemed to be arranged in a parallel fashion (Fig. 7). One of the reasons for this observation may be due to the fact that cells on PT surfaces are more easily able to spread and migrate to one another, whereas cells seeded on SLA surfaces are more

confined by their three-dimensional space due to the enhanced roughened surface.

The enhanced ECM matrix on titanium surfaces cultured with EMD may play a number of possible roles for enhanced initial soft tissue attachment to implant surfaces. As the results from the present study seem to favor soft tissue colonization onto implant surfaces, it suggests that a faster formation of a three-dimensional extracellular matrix around the implant surface may also prevent bacterial contamination within the bone-to-implant region of the titanium surface. It has been suggested that approximately 50% of implants are lost within the first year of placement, sometimes even prior to loading, and one may expect that bacterial contamination may play a role in this observation. Not only is EMD able to speed up wound healing/closure, but it has also been reported that EMD does possess some form of anti-microbial effect which is likely due to the carrier system [71–75]. Furthermore, the use of EMD significantly increases the expression of VEGF, a known angiogenic growth factor capable of inducing vascularization. As the soft tissues around implants are much less vascularized when compared to natural teeth due to the loss of the periodontal ligament blood supply, it becomes vital that enough tissue oxygenation be present for the regeneration of host tissues. As such, the ability for EMD to rapidly increase angiogenesis is a known advantage that may indirectly be responsible for its wound healing capabilities. It has also been reported that a growing concern for peri-implantitis is more routinely being observed in clinical practice [76]. Froum et al. has been able to partially regenerate a loss of clinical attachment around implants via a biological approach utilizing a combination of a membrane, bone graft, and growth factors including EMD [77]. Future research in this field is necessary to fully characterize the full potential of EMD on the soft tissue integration and regeneration of gingival tissues around titanium implants.

Conclusions

The results from the present study demonstrate the effects of EMD on gingival fibroblast cell behavior when seeded on PT and SLA titanium surfaces. Firstly, EMD was able to significantly increase cell spreading of cells seeded on PT and SLA surfaces at various early time points. Furthermore, EMD increased cell proliferation of gingival fibroblasts at 3 and 5 days on SLA surfaces and 5 days for PT surfaces when compared to their respective controls without EMD. Real-time PCR experiments demonstrated that EMD was able to significantly upregulate mRNA levels of fibronectin, VEGF-A, and collagen 1. Furthermore, EMD induced ECM synthesis as assessed by immunofluorescence staining of collagen 1 at 2 weeks post-seeding. The findings from the present investigation suggest that EMD may improve the speed of soft tissue healing around titanium implant collars. Future animal research is required to fully characterize the effects of EMD on soft tissue wound healing around implants.

Conflict of interest The authors report no conflict of interest. All materials and funding for the project were provided by The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University.

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