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Effects of enamel matrix derivative on periodontal wound healing in an inflammatory environment in vitro

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Abstract

Aim: This in vitro study was established to investigate whether the regenerative capacity of periodontal ligament (PDL) cells in the presence of enamel matrix derivative (EMD) is modulated by inflammation.

Materials and Methods: PDL cells were grown in the presence or absence of EMD under normal and inflammatory conditions for up to 14 days. In order to mimic an inflammatory environment, cells were incubated with interleukin (IL)-1β. Cells were also exposed to transforming growth factor (TGF)-β1 and insulin-like growth factor (IGF)-1 under both conditions. For analysis of wound healing, an in vitro wound fill assay was used. The synthesis of growth factors, markers of proliferation, and osteogenic differentiation, as well as collagen was studied by real-time polymerase chain reaction, enzyme-linked immunoassay, and immunoblotting. Mineralization was assessed by alizarine red S and von Kossa staining.

Results: EMD stimulated significantly the in vitro wound fill rate, cell proliferation and adhesion, synthesis of growth factors, and collagen, as well as mineralization. In the presence of IL-1β, these EMD effects were significantly reduced. IL-1β also inhibited significantly the wound fill rate induced by TGF-β1 and IGF-1.

Conclusions: Critical PDL cell functions that are associated with periodontal regeneration are reduced in an inflammatory environment.

Key words: collagen; enamel matrix derivative; inflammation; insulin-like growth factor; periodontium; transforming growth factor; wound healing

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Periodontitis is characterized by an inflammatory process caused by bacterial components and products, which trigger the production of inflammatory molecules by infiltrating and resident cells in the periodontium. These inflammatory mediators can result in progressive destruction of the periodontal tissues, and thereby, in pocket formation and tooth loss (Oates & Cochran 1996, Preshaw et al. 2004, Tatakis & Kumar 2005, Van Dyke & Sheilesh 2005). Periodontal therapy is aimed to arrest the inflammatory process by removing the subgingival biofilm and creating a local environment and microflora, which are compatible with periodontal health. In addition, periodontal treatment is performed to reduce periodontal pockets and improve clinical attachment levels. Conventional periodontal treatment comprises non-surgical or surgical debridement, sometimes applied in combination with antibiotics (Heitz-Mayfield 2005), and achieves healing mainly by repair (Garrett 1996).

One treatment approach to accomplish healing by regeneration is the application of enamel matrix derivative (EMD) during periodontal surgery. EMD is extracted from developing embryonic enamel of porcine origin and contains hydrophobic enamel matrix proteins (Hammarstrom 1997). Significant evidence has been provided that EMD promotes regeneration of periodontal tissues and improvement of clinical attachment level and probing pocket depth (Pontoriero et al. 1999,

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Enamel matrix derivative (EMD) was provided by Straumann AG, Basel, Switzerland.

Tonetti et al. 2002, Cochran et al. 2003, Giannobile & Somerman 2003, Jepsen et al. 2004, Venezia et al. 2004, Trombelli & Farina 2008, Esposito et al. 2009). Although the complex role of EMD in periodontal regeneration is still incompletely understood, it has been revealed that EMD affects cell proliferation, attachment and apoptosis, synthesis of growth factors and matrix molecules, as well as mineralization (Bosshardt 2008). Transforming growth factor (TGF)-β-like and bone morphogenetic protein (BMP) activities were found in several fractions of EMD (Suzuki et al. 2005). In addition, EMD has been shown to upregulate the synthesis of growth factors, such as TGF-β1 and insulin-like growth factor (IGF)-1 (Okubo et al. 2003, Parkar & Tonetti 2004). Therefore, it has been suggested that the cementum and osteopromotive activities of EMD are induced at least in part by such growth factors (Takayama et al. 2005).

Wound healing is characterized by inflammation, matrix deposition, and remodelling (Aukhil 2000) but whether and how much inflammation is needed for optimal tissue repair are as yet unknown (Eming et al. 2007, 2009). Most in vitro studies on EMD were performed in the absence of bacterial infection and inflammation, which has allowed unraveling the full potential of EMD under optimal conditions. However, taking into account that EMD is often applied in inflamed conditions due to a residual periodontal infection, there is an ultimate need to also assess the effects of EMD under inflammatory conditions. Therefore, this in vitro study was established to investigate whether the regenerative capacity of periodontal ligament (PDL) cells in the presence of EMD is modulated by inflammation. A better understanding of the effects of regenerative molecules in an inflammatory environment may result in more powerful treatment strategies in the future.

Materials and Methods

Cell culture

Human PDL cells were harvested from periodontally healthy teeth that had to be extracted for orthodontic reasons. Informed parental consent and approval of the Ethics Committee of the University of Bonn were obtained. The cells from the middle third of the tooth roots were cultured in Dulbecco's minimal essential medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS, Invitrogen), 100 units penicillin, and 100 µg/ml streptomycin (Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO_2. Cells from six different individuals were phenotyped with the use of osteogenic markers, as described by Basdra & Komposch (1997) and Chou et al. (2002), and used between passages 3 and 5. PDL cells were seeded (50,000 cells/well) on cell culture plates and grown to 80% confluence. One day before the experiment, the FBS concentration was reduced to 1%. Medium was changed every other day.

In order to determine how the beneficial actions of EMD pertinent to regenerative periodontal healing are affected by inflammation, cells were cultured in the presence and absence of EMD (kindly provided by Straumann AG) under normal and inflammatory conditions. EMD was used at a concentration of 0.1 mg/ml. This concentration has been used in several in vitro studies by different investigators (Hoang et al. 2000, Rincon et al. 2003, Parkar & Tonetti 2004, Schwarz et al. 2004). Because several studies have revealed that interleukin (IL)-1β is significantly increased in gingiva and gingival crevicular fluid (GCF) at inflamed sites, cells were incubated with this cytokine to simulate an inflammatory environment (Honig et al. 1989, Hou et al. 1994, Preiss & Meyle 1994, Mathur et al. 1996). IL-1β has also been used by other investigators to mimic inflammation in vitro and was applied at concentrations (0.1–10 ng/ml; Calbiochem, San Diego, CA, USA), which are in the range of levels usually found in GCF of periodontally diseased patients (Long et al. 2002, Agarwal et al. 2003). Cells were also exposed to TGF-β1 (10 ng/ml; PromoCell, Heidelberg, Germany) and IGF-1 (25 ng/ml; PromoCell) in the presence or absence of IL-1β. In order to unravel intracellular mechanisms underlying the effects of EMD and/or IL-1β on gene expression, cells were pre-incubated with a SMAD1/5/8 inhibitor (dorsomorphin, 5 μM; Calbiochem) or a c-Jun *N*-terminal kinase (JNK) inhibitor (SP600125, 10 μM; Calbiochem) 1 h before the experiments.

Real-time polymerase chain reaction (PCR)

RNA was extracted using an RNA extraction kit (Qiagen, Hilden, Germany), and a total of 1 µg of RNA was reverse transcribed using iScriptTM Select cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) at 42°C for 80 min. followed by 85°C for 5 min. Expression of IGF-1, TGF-β1, and its receptors (TGF-βR1, TGF-βR2), vascular endothelial growth factor (VEGF), BMP2, Ki67, proliferating cell nuclear antigen (PCNA), collagen type I (COL1), and runt-related transcription factor (RUNX) 2 was detected by real-time PCR using the iCycler iQ detection system (Bio-Rad Laboratories), SYBR Green (Bio-Rad Laboratories), and specific primers (QuantiTect Primer Assay; Qiagen). One microliter of cDNA was amplified as a template in a 25 μl reaction mixture containing 12.5 μl of 2 × QuantiFast SYBR Green PCR Master Mix (Qiagen), 2.5 µl of primers, and deionized water. The mixture was heated initially at 95°C for 5 min. and then followed by 40 cycles with denaturation at 95°C for 10 s, and combined annealing/extension at 60°C for 30 s. GAPDH was used as an endogenous control. Following amplification, melt curve protocols were performed to ensure that primer–dimers or non-specific products had been eliminated or minimized. The data were analysed by the comparative threshold cycle method (Livak & Schmittgen 2001).

ELISA

The levels of active TGF-β1 in the supernatants of PDL cells were analysed by a commercially available enzyme-linked immunoassay (ELISA) kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. The absorbance was measured with a microplate reader (PowerWave x, BioTek Instruments, Winooski, VT, USA) at 450 nm. The data were normalized by the cell number, which was measured with an automatic cell counter (Moelab, Hilden, Germany).

In vitro wound fill assay

In order to study the wound fill rate in vitro, we used an in vitro wound-healing model, which has been well described and successfully utilized by other investigators (Hoang et al. 2000, Lackler et al. 2000, Katz et al. 2002, Fang & Svoboda 2005, Jia & Turek 2005). Briefly, PDL cells were grown until confluence and 3 mm wide wounds in the cell monolayers were created by a sterile instrument in a standardized man-

ner. The wounded cell monolayers were grown in the presence or absence of EMD, IGF-1, and TGF-β1 under normal or inflammatory conditions for 3 days. At every day, the gaps of the monolayers were documented by inverse microscopy (Axiovert 25 C, × 5 objective, Zeiss, Oberkochen, Germany) and digital photography (Kodak DC 290, Stuttgart, Germany). The widths of the gaps were then measured and analysed with special software (Alpha DigiDoc 1000, Alpha Innotech, San Leandro, CA, USA). In order to determine the percentage of wound fill, the gap widths at 1, 2, and 3 days were related to the gap widths measured at baseline.

Cell adhesion and cell number

In order to determine the EMD-stimulated adhesion of PDL cells under normal and inflammatory conditions, cells were allowed to attach to the well surface of six-well culture plates during a 4-h incubation with EMD in the presence or absence of IL-1β. Then, cell cultures were washed twice with 500 μl of PBS (PAA Laboratories, Pasching, Austria), and subsequently, incubated with 120 μl of 1% trypsin/EDTA (PAA Laboratories) for 5 min. The trypsin/EDTA solution was neutralized by the addition of an equal volume of DMEM supplemented with 10% FBS. The cell suspension was collected and centrifuged at 500 *g* at room temperature (RT) for 10 min. The supernatant was discarded and the pellet was re-suspended in 20 μl of PBS. Finally, the cells were counted with a haemacytomer (Fisher Scientific, Schwerte, Germany).

Western blot analysis

Synthesis of COL1 was analysed from whole lysate (20 to 40 μg protein) of cells treated with EMD in the presence or absence of IL-1β for 3 days. Cells were washed twice with ice-cold PBS and collected in RIPA buffer (Sigma, Dahlkirchen, Germany) supplemented with protease inhibitors (Sigma). The protein concentration was measured using a BCA Protein Assay Reagent Kit (Pierce, Bonn, Germany) and a microplate reader (see above) at 562 nm. Equal amounts of protein were resolved through SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes blocked with 5% non-fat milk were probed with a specific rabbit polyclonal antibody against collagen α1 type 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The binding of the primary antibody was revealed with a horseradish peroxidase (HRP)-labelled polyclonal goat anti-rabbit secondary antibody (Jackson ImmunoResearch, Suffolk, UK). Lightning chemiluminescence reagent (Pierce) was used as an HRP substrate. All blots were re-probed with monoclonal anti-mouse β-actin antibodies (Abcam, Cambridge, UK) to assure equal input of proteins.

Alizarin red S staining

Accumulation of calcium in the PDL cell matrix was analysed by alizarine red S (Merck, Darmstadt, Germany) staining and cetylpyridinium chloride (CPC; Sigma) according to the method described by Reinholz et al. (2000) following 14 days of treatment. Briefly, PDL monolayers were washed twice with PBS and fixed in 4% paraformaldehyde (Merck) at RT for 10 min. Then, paraformaldehyde was removed and cells were washed twice with an excess of dH_2O. Afterwards, 500 μl of 40 mM alizarine red S (pH 4.2) was added to each well. The plates were then incubated at RT for 15 min. with gentle agitation. Then, wells were washed five times with 500 μl of dH_2O while shaking for 5 min. Afterwards, the stained PDL monolayers were visualized and analysed by microscopy and digital photography (Axioskop 2, Axiocam MRc, Axiovision 4.7/AutMess, Zeiss). In order to quantify the mineralized nodules, dH_2O was replaced with 10% (w/v) CPC in 10 mM Na_2PO_4 (pH 7.0). The plates were incubated at RT for 15 min. with agitation. The cell monolayers were then completely detached from the well surface. The cell suspension was collected and centrifuged at 20,000 *g* for 10 min. The supernatants were transferred into a 96-well plate to measure the absorbance at 562 nm with a microplate reader (see above).

Von Kossa-staining

PDL cells seeded on cover slips (Fisher Scientific) were treated with EMD in the presence or absence of IL-1β for 14 days. At the end of experiments, cells were washed twice with PBS (PAA Laboratories) and fixed with 4% paraformaldehyde (Sigma) at RT for 10 min. Afterwards, cells were washed twice with dH2O, and subsequently, treated with 5% silver nitrate solution (Merck) at 4°C for 40 min. Then, the coverslips were washed with dH2O and incubated with 1% pyrogallol (Merck) at RT for 5 min. After washing, unreacted silver was removed with 5% sodium thiosulphate (Merck) at RT for 5 min. Coverslips were rinsed again with dH2O and counterstained with 0.1% nuclear fast red solution at RT for 10 min. After a final washing, the coverslips were dehydrated through 100% ethanol (Sigma) for 2 min. and cleared in xylene for another 2 min. Finally, the coverslips were mounted (DePeX, Serva Feinbiochemica, Heidelberg, Germany) and air-dried for histomorphometric analysis, using a transmitted light microscope, a digital high-resolution microscopy camera, and a special software (Axioskop 2, Axiocam MRc, Axiovision 4.7/AutMess, Zeiss).

Statistical analysis

The SPSS 14.0 software was used for statistical analysis. For quantitative analysis of the mRNA expression, mean values and standard errors of the mean were calculated. Experiments were repeated at least twice. For statistical analysis, parametric and non-parametric tests were applied.

Results

Effect of an inflammatory environment on the EMD-enhanced wound fill rate

The re-population of the periodontal wound with PDL cells after surgery is critical to regenerative healing. We therefore used an in vitro wound-healing assay to determine the effect of IL-1β on the wound fill rate in the absence and presence of EMD, IGF-1, or TGF-β1. At all days, EMD stimulated significantly the re-population of the wounded areas as compared with the control (Fig. 1a). Similarly to the effect of EMD, the wound fill rate was significantly increased by IGF-1 and TGF-β1 at all days and from day 2 on, respectively (Fig. 1a). However, in contrast to the stimulation of the wound fill rate by these molecules, the wound closure was delayed in the presence of IL-1β, which was significant at day 3 (Fig. 1a).

As shown in Fig. 1b, the beneficial effect of EMD on the wound fill rate was inhibited dose-dependently by IL-1β, i.e., the strongest inhibitory effect was exerted by the highest concentration

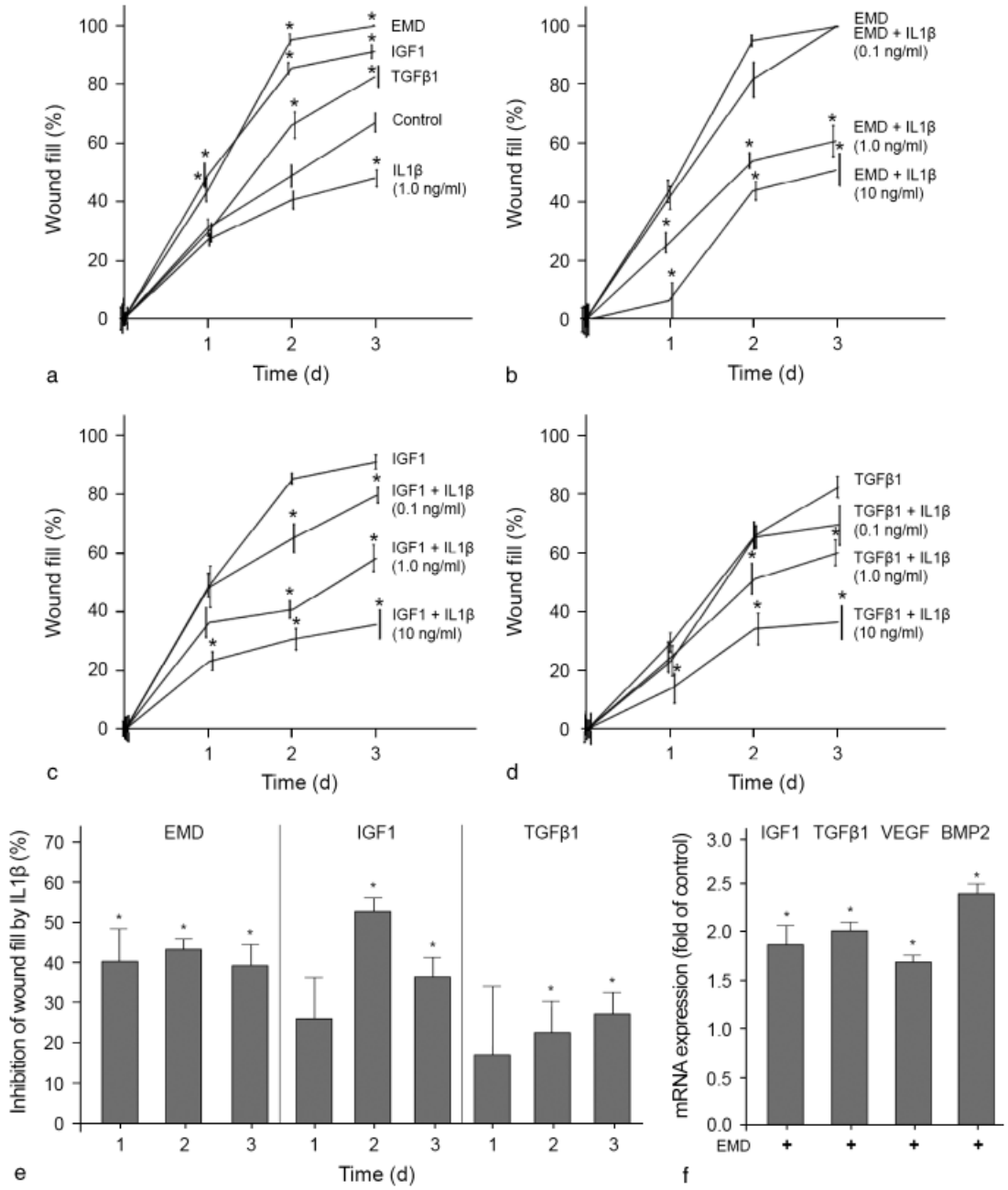


Fig. 1. Effect of interleukin (IL)-1β on periodontal ligament cell wound fill rate stimulated with enamel matrix derivative (EMD), insulin-like growth factor (IGF)-1, or transforming growth factor (TGF)-β1. The wound closure, i.e. the percentage of fill of the initially cell-free zones created by wounding, were analysed after 1, 2, and 3 days. Wounded cell monolayers were incubated with EMD, IGF-1, TGF-β1, or IL-1β, whereas wounded monolayers in the absence of these molecules served as control (a). In addition, wounded monolayers were stimulated with EMD (b), IGF-1 (c), or TGF-β1 (d) in the presence and absence of various concentrations of IL-1β (0.1, 1.0, and 10 ng/ml). Mean values ± SEM were calculated ($n = 12$/group and time point). For statistical analysis, one-way analysis of variance, followed by Dunnett's test as post hoc, was used. *Significantly ($p < 0.05$) different from control (a) or significantly ($p < 0.05$) different from IL-1β-untreated cells (b–d). Inhibition of wound closure by IL-1β (1 ng/ml) in EMD-, IGF-1-, or TGF-β1-stimulated cell cultures from Fig. 1b–d (e). Cell cultures incubated with these stimulants in the absence of IL-1β served as control. *Significantly ($p < 0.05$) different from control. Expression of growth factors [insulin-like growth factor (IGF)1, TGF-β1, vascular endothelial growth factor (VEGF), and bone morphogenetic protein (BMP2)] following 1 day of incubation with EMD (f). Untreated cells served as control. Mean values ± SEM were calculated ($n = 6$/group). For statistical analysis, Student's *t*-test was applied. *Significantly ($p < 0.05$) different from control.

of IL-1β, whereas IL-1β at low concentration did not exert a significant effect. Like in EMD-treated cells, the stimulatory effect of IGF-1 and TGF-β1 on the wound fill rate was significantly abrogated by IL-1β in a dose-dependent manner (Fig. 1c and d). IL-1β at a concentration of 1 ng/ml inhibited the wound closure in EMD-treated cells by 40%, 43%, and 41%, in IGF-1-stimulated cells by 29%, 53%, and 38%, and in TGF-β1-incubated cells by 13%,

25%, and 27% after 1, 2, and 3 days, respectively (Fig. 1e).

Upregulation of IGF-1 and TGF-β1 by EMD

Because it has been suggested that EMD effects are partially mediated by IGF-1 and TGF-β1, we studied the mRNA expression of these growth factors in the presence or absence of EMD. Following 1 day of incubation with EMD, cells expressed significantly higher mRNA levels for IGF-1, TGF-β1, VEGF, and BMP2 than the control (Fig. 1f).

Effect of an inflammatory environment on the EMD-stimulated PDL cell proliferation

Because the wound fill rate is determined at least in part by the number of PDL cells available for the re-population of the wound, we investigated the effect of IL-1β on the EMD-stimulated PDL cell proliferation. Cells were cultured in the presence and absence of EMD under normal and inflammatory conditions for 8 days. No significant differences between groups were observed at 1 day (Fig. 2a). At 2 days, EMD caused an increase in the cell

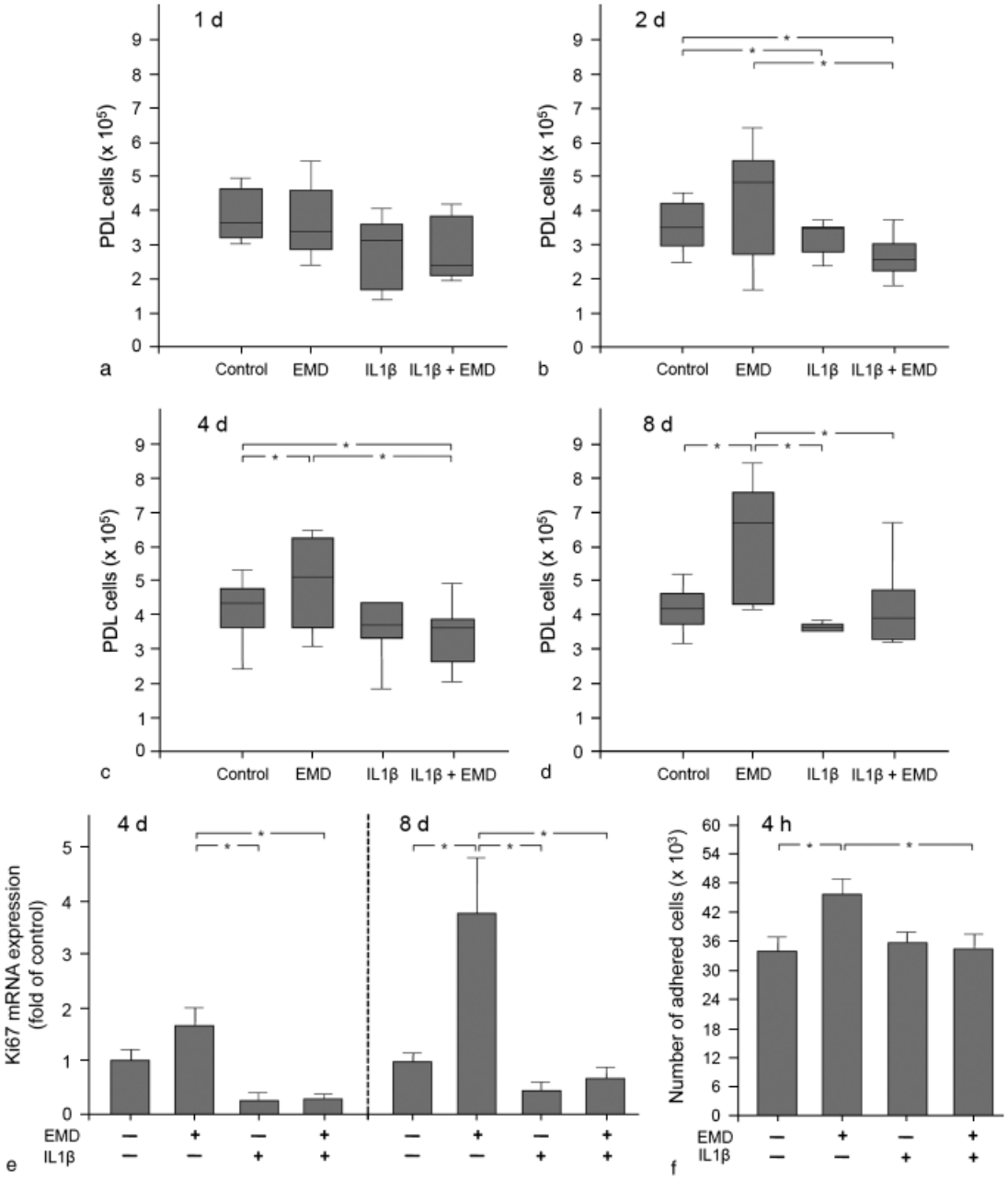


Fig. 2. Effect of interleukin (IL)-1β on the enamel matrix derivative (EMD)-stimulated increase in periodontal ligament (PDL) cell numbers after 1, 2, 4, and 8 days (a–d). Cells were incubated with EMD and/or IL-1β. Untreated cells served as control. For statistical analysis, the Wilcoxon test was used ($n = 6$/group and time point). *Significant ($p < 0.05$) difference between groups. Effect of IL-1β on the EMD-stimulated Ki67 mRNA expression at 4 and 8 days (e) and cell adhesion at 4 h (f). Cells were incubated with EMD and/or IL-1β. Untreated cells served as control. Mean values ± SEM were calculated ($n = 6$/group). For statistical analysis, one-way analysis of variance and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups.

number in the absence of IL-1β, but not under inflammatory conditions. The cell number of EMD-treated cultures differed significantly between normal and inflammatory conditions (Fig. 2b). The stimulatory effect of EMD on the cell number in normal conditions was even more pronounced and significantly different from the control at 4 and 8 days (Fig. 2c and d). By contrast, no such stimulatory effect of EMD on cell numbers was observed, when cells were simultaneously exposed to an inflammatory environment (Fig. 2c and d). In addition, EMD upregulated the Ki67 mRNA expression, which was significantly abrogated by IL-1β at 4 and 8 days (Fig. 2e). Similar findings were observed for PCNA (data not shown). Taken together, these results suggest that the beneficial effects of EMD on cell number and proliferation, respectively, are diminished in the presence of IL-1β.

Cell adhesion in the presence of EMD in an inflammatory environment

Periodontal wound healing also requires adhesion of PDL cells to the tooth surface, extracellular matrix, and other cells. We therefore sought to analyse whether IL-1β would interfere with the actions of EMD on PDL cell adhesion. At 4 h, EMD caused a significant increase in the number of adhered cells as compared with the control, but this stimulatory effect of EMD was completely suppressed in the presence of IL-1β (Fig. 2f).

Actions of IL-1β on the EMD-upregulated expression of growth factors

Because TGF-β1 and VEGF are induced by EMD and play an important role in wound healing, we also studied the EMD-induced synthesis of these growth factors in the presence or absence of IL-1β. EMD enhanced significantly TGF-β1 mRNA expression at 1 and 6 days (Fig. 3a). When cells were exposed to EMD under inflammatory condition, the stimulatory effect of EMD on TGF-β1 mRNA expression was significantly reduced at both time points (Fig. 3a). At 1 and 6 days, EMD also caused a significant upregulation of VEGF (Fig. 3b). IL-1β had no effect on the VEGF expression at 1 day, but significantly increased VEGF mRNA levels at 6 days (Fig. 3b). At 1 day, the EMD-upregulated VEGF expression was again significantly reduced by IL-1β, whereas no significant effect of IL-1β on the EMD-induced VEGF expression was observed at 6 days (Fig. 3b). The inhibitory effect of IL-1β on the EMD-induced stimulation of TGF-β1 synthesis was also found at a protein level, as analysed by ELISA. At 1 day, EMD-stimulated cells in the absence and presence of IL-1β produced 28.38 ± 0.22 ng/10^6 cells and 25.12 ± 0.20 ng/10^6 cells, respectively. At 2 days, EMD-treated cells in the absence and presence of IL-1β synthesized 15.45 ± 0.10 and 11.43 ± 0.30 ng/10^6 cells, respectively. The differences between both groups at each time point were significant.

Next, we sought to determine the mechanisms underlying the stimulatory effects of EMD and the inhibitory actions of IL-1β. When cells were stimulated with EMD in the presence of dorsomorphin, a specific inhibitor of BMP signalling through the SMAD pathway, the EMD-upregulated TGF-β1 and VEGF expression was significantly diminished at 1 day (Fig. 3c). Furthermore, the inhibition of the EMD effects on TGF-β1 and VEGF by IL-1β was partially abrogated in the presence of SP600125, a potent and selective inhibitor of JNK (Fig. 3d).

Because EMD-induced TGF-β1 may mediate critical EMD effects associated with wound healing, we also studied the effect of IL-1β on TGF-β1 actions. TGF-β1 caused a significant upregulation of its own expression as well as the expression of VEGF, PCNA, and COL1 at 1 day (Fig. 3e). Interestingly, the TGF-β1-induced stimulation of these molecules was again significantly inhibited by IL-1β (Fig. 3e).

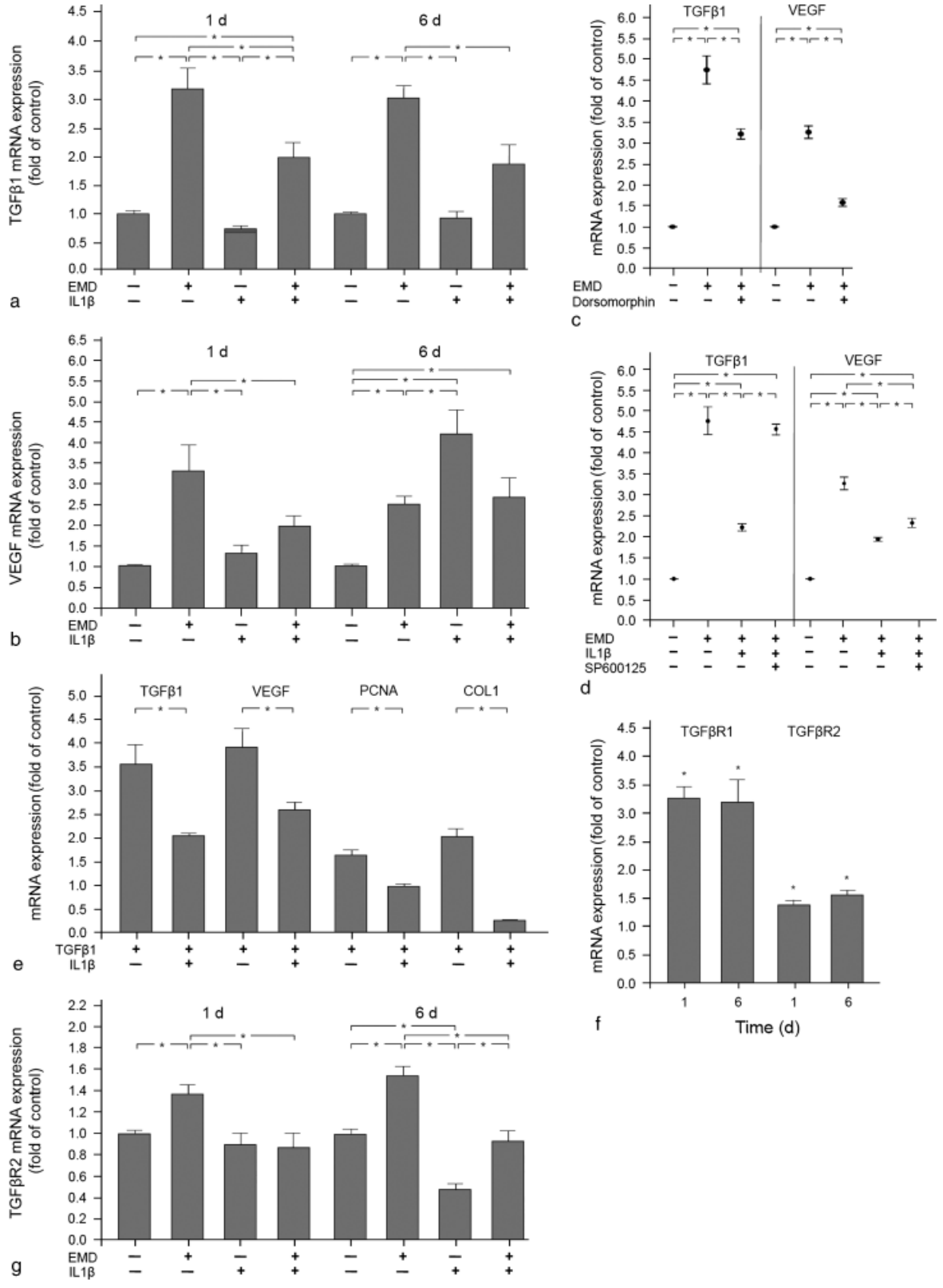
The effects of TGF-β1 depend on the availability of its receptors. Therefore, we also investigated whether EMD stimulates the expression of TGF-βR1 and TGF-βR2 in PDL cells. Cells that were exposed to EMD expressed significantly more TGF-βR1 and TGF-βR2 than control cells at 1 and 6 days (Fig. 3f). The EMD-upregulated TGF-βR2 mRNA expression was completely inhibited by IL-1β at both time points (Fig. 3g), whereas no regulatory effect of IL-1β on the EMD-stimulated TGF-βR1 expression was found (data not shown).

Regulation of the EMD-stimulated matrix synthesis by IL-1β

Periodontal wound healing requires synthesis and remodelling of the extracellular matrix, which consists mainly of COL1. Therefore, we also studied the effect of EMD on COL1 in the presence and absence of IL-1β. Following 1 and 6 days of incubation with EMD, cells expressed significantly more COL1 mRNA than control (Fig. 4a). In contrast, IL-1β caused a remarkable downregulation of the COL1 mRNA

Fig. 3. Effect of interleukin (IL)-1β on enamel matrix derivative (EMD)-induced mRNA expression of transforming growth factor (TGF)-β1 (a) and vascular endothelial growth factor (VEGF) (b) at 1 and 6 days. Cells were incubated with EMD and/or IL-1β. Untreated cells served as control. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, one-way analysis of variance (ANOVA) and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Involvement of SMAD signalling in actions of EMD on TGF-β1 and VEGF expression (c). Cells were incubated with or without 5 μM dorsomorphin for 1 h and then stimulated with EMD. Untreated cells served as control. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Involvement of c-Jun *N*-terminal kinase signalling in the regulation of EMD actions by IL-1β (d). Cells were incubated with or without 10 μM SP600125 for 1 h and, subsequently, stimulated either with EMD alone or a combination of EMD and IL-1β. Untreated cells served as control. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Effect of IL-1β on TGF-β1-induced mRNA expression of TGF-β1, VEGF, proliferating cell nuclear antigen (PCNA), and collagen type I (COL1) at 1 day (e). Cells were incubated either with TGF-β1 alone or a combination of TGF-β1 and IL-1β. Untreated cells served as control. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Effect of EMD on TGF-βR1 and TGF-βR2 expression at 1 and 6 days (f). Cells were cultured in the presence and absence (control) of EMD. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, Student's *t*-test was applied. *Significant ($p < 0.05$) difference between EMD-treated cells and control. Effect of IL-1β on EMD-induced TGF-βR2 mRNA expression at 1 and 6 days (g). Cells were incubated with EMD and/or IL-1β. Untreated cells served as control. Mean values $\pm$ SEM were calculated ($n = 6$/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups.

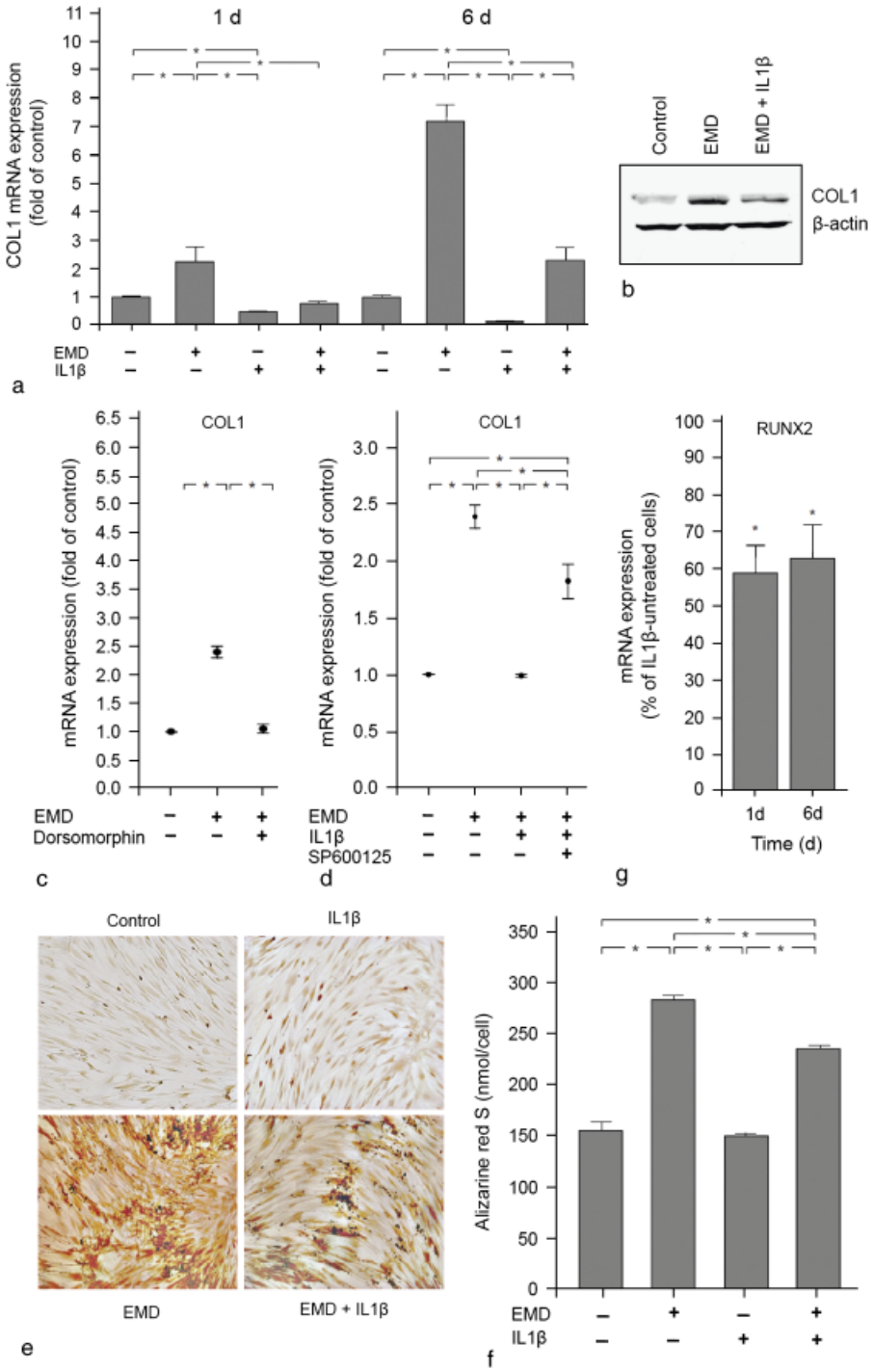
expression at these time points. Furthermore, the EMD-stimulated COL1 mRNA expression was significantly reduced in the presence of IL-1β, which was also confirmed at the protein level, as determined by immunoblotting (Fig. 4a and b). The EMD-upregulated CO L1 expression was completely blocked, when EMD-treated cells were exposed to dorsomorphin for 1 day (Fig. 4c). In addition, the IL-1β-induced inhibition of the EMD-stimulated COL1 expres-



sion was reduced in the presence of SP600125 (Fig. 4d).

Calcium deposition induced by EMD under inflammatory condition

Formation of new bone is a major component of periodontal regeneration. Incubation of PDL cells with EMD for 2 weeks stimulated calcium deposition in the extracellular matrix, as demonstrated by alizarin red S staining (Fig. 4e and f). Although EMD also induced calcium deposition in the presence of IL-1β, the stimulatory effect of EMD was significantly reduced, as compared with normal conditions (Fig. 4e and f). These findings were confirmed by von Kossa staining, which revealed an IL-1β-induced reduction of calcium deposition by 34.88 $\pm$ 9.62%. Finally, we analysed the expression of RUNX2, a typical marker of osteogenic differentiation, in EMD-treated PDL cells in the presence and absence of IL-1β. At 1 and 6 days, the EMD-induced RUNX2 mRNA



expression was significantly inhibited by approximately one-third in an inflammatory environment, as compared with normal condition (Fig. 4g).

Discussion

EMD is widely used to reconstitute periodontal structures in patients afflicted with periodontitis and has been shown to stimulate properties of PDL cells, which are clearly associated with periodontal regeneration (Boshardt 2008). That the beneficial effects of EMD on PDL cell migration, proliferation, adhesion, matrix synthesis, and osteogenic differentiation are reduced under inflammatory conditions is a novel finding and of major clinical importance, because it implicates that an efficient anti-infectious and anti-inflammatory periodontal treatment is critical before the application of EMD.

In our experiments, EMD caused an increase in cell numbers and an upregulation of markers of cell proliferation, which is in accordance with other studies, which used the same or different assays (Gestrelius et al. 1997, Cattaneo et al. 2003, Pischon et al. 2006, Heng et al. 2007, Rodrigues et al. 2007). Interestingly, the EMD-stimulated increase in cell numbers was completely abrogated by IL-1β. In addition, the EMD-upregulated gene expression of proliferation markers was also diminished in an inflammatory environment, suggesting that inflammatory signals inhibit PDL cell proliferation.

Following periodontal surgery, the periodontal wound space needs to be re-populated with PDL cells to achieve periodontal regeneration. EMD has been shown to accelerate wound-fill rates in vitro due to proliferation and migration. In a number of studies, an in vitro wound-healing model like the one in our study has been used. This model is characterized by scraping a 3–4-mm-wide cell-free zone in cell monolayers and analysing cell migration into the cell-free zone by histomorphometry (Hoang et al. 2000, Chong et al. 2006). Exposure of PDL cells to EMD accelerated the wound-fill rates (Hoang et al. 2000, Rincon et al. 2003, Chong et al. 2006, Rodrigues et al. 2007), as it was found in our experiments. In contrast to EMD, IL-1β caused a delay of the in vitro wound closure. Furthermore, IL-1β antagonized the stimulatory effect of EMD on wound closure in a dose-dependent manner, i.e., the most pronounced inhibition of the EMD-stimulated wound fill rate was observed at the highest concentration of IL-1β. TGF-β1 and IGF-1, both growth factors produced by PDL in response to EMD, promoted the wound closure in PDL cell monolayers similar to EMD, suggesting that the EMD-induced stimulation of wound healing may be at least partially mediated by these molecules (Okubo et al. 2003). However, more importantly, the positive influence of these growth factors on the wound fill rate was diminished dose-dependently in the presence of IL-1β, as found in EMD-treated cultures. Again, these findings suggest that the full regenerative capacity of PDL cells can only be achieved in the absence of inflammation. Notably, it has been reported that migration and proliferation of EMD-stimulated PDL cells are markedly diminished by *P. gingivalis*, which is in line with our findings and emphasizes again the need for an efficient anti-infectious and anti-inflammatory periodontal treatment before the application of EMD (Inaba et al. 2004, 2005).

In order to reconstitute the periodontium, PDL cells have to attach to the root surface, other surrounding cells, and the extracellular matrix. Although opposite results have also been reported, EMD causes an increase in PDL cell adhesion (Gestrelius et al. 1997, Lyngstadaas et al. 2001; Palioto et al. 2004; Rodrigues et al. 2007). In our experiments, the PDL cell adhesion was also significantly enhanced by EMD, but the stimulatory effect was completely absent in the presence of IL-1β.

The cellular properties such as cell proliferation, migration, and adhesion are tightly regulated by growth factors such as VEGF and TGF-β1, produced by structural and inflammatory cells in the periodontium. We therefore sought to determine how these growth-promoting factors are expressed in EMD-treated cells under normal and inflammatory conditions. EMD induced an initial upregulation of both growth factors in the absence and presence of IL-1β, even though the stimulatory effect of EMD on the VEGF and TGF-β1 gene expression was reduced in an inflammatory environment. These results are in accordance with findings from studies of other investigators, who also reported an enhanced expression of TGF-β1 in PDL cells exposed to EMD (Van der Pauw et al. 2000, Lyngstadaas et al. 2001, Okubo et al. 2003, Inaba et al. 2004, Parkar & Tonetti 2004). However, according to our study, the EMD-induced TGF-β1 levels seem to be modulated by inflammation.

TGF-β1 actions depend on binding of TGF-β to its receptors. TGF-βR1 and 2

Fig. 4. Effect of interleukin (IL)-1β on enamel matrix derivative (EMD)-induced collagen type I (COL1) mRNA expression at 1 and 6 days (a). Periodontal ligament cells were exposed to EMD and/or IL-1β. Untreated cells were used as control. Mean values ± SEM were calculated (n = 6/group). For statistical analysis, one-way analysis of variance (ANOVA) and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Effect of IL-1β on COL1 synthesis at 3 days, as analysed by immunoblotting (b). Cells were stimulated with EMD in the absence and presence of IL-1β. Untreated cells served as control. Involvement of SMAD signalling in actions of EMD on COL1 mRNA expression (c). Cells were incubated with or without 5 μM dorsomorphin for 1 h and then stimulated with EMD. Untreated cells served as control. Mean values ± SEM were calculated (n = 6/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Involvement of c-Jun *N*-terminal kinase signalling in the regulation of EMD actions by IL-1β (d). Cells were incubated with or without 10 μM SP600125 for 1 h and, subsequently, stimulated either with EMD alone or a combination of EMD and IL-1β. Untreated cells served as control. Mean values ± SEM were calculated (n = 6/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Effect of IL-1β on calcium deposition in cell cultures (e and f). Cells were incubated with EMD and/or IL-1β. Untreated cells served as control. The calcium deposition was analyzed by alizarin red S-staining, visualized by microscopy (e) and quantified by elution with cetylpyridinium chloride (f) after 2 weeks. Mean values ± SEM were calculated (n = 6/group). For statistical analysis, one-way ANOVA and the Tukey post hoc test were applied. *Significant ($p < 0.05$) difference between groups. Effect of IL-1β on EMD-induced RUNX2 mRNA expression at 1 and 6 days (g). EMD-stimulated cells were cultured in the presence and absence (control) of IL-1β. Mean values ± SEM were calculated (n = 6/group). For statistical analysis, Student's t-test was applied. *Significant ($p < 0.05$) difference between IL-1β-treated and -untreated cells.

are protein kinases characterized by an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic serine–threonine kinase domain. After binding of TGF-β to TGF-βR2, TGF-βR1 is recruited into a heterotetrameric receptor complex, and subsequently, phosphorylated on serine residues by TGF-βR2, which finally activates the SMAD signal transduction pathway (Prime et al. 2004). In our experiments, PDL cells expressed receptors for TGF-β1, which confirms findings by other investigators (Chien et al. 1999, Gao et al. 1999, Parkar et al. 2001, Murakami et al. 2003). As a novel finding, we also observed that EMD upregulates TGF-βRs and that the EMD-stimulated TGF-βR2 mRNA expression is regulated by IL-1β.

EMD is also involved in the regulation of matrix synthesis by periodontal cells, which constitutes an important component of the periodontal wound healing (Silverio-Ruiz et al. 2007). Therefore, we wondered if EMD would upregulate the COL1 synthesis in PDL cells and if IL-1β would again interfere with the EMD-regulated COL1 production. When cells were subjected to treatment with EMD, the synthesis of COL1 was enhanced. Because EMD-induced growth factors like TGF-β1 are known to stimulate production of COL1, the EMD-enhanced COL1 synthesis may be partially mediated by TGF-β1, even though it was not analysed in this study. However, this speculation receives further support by the fact that the EMD-stimulated COL1 synthesis was diminished under inflammatory conditions as was the EMD-induced TGF-β1 upregulation.

PDL cells have or acquire an osteoblastic phenotype, and a number of studies have proven that EMD promotes the expression of markers associated with osteogenic differentiation, such as production of osteocalcin and increased alkaline phosphatase activity, and stimulates mineral nodule formation (Gestrelius et al. 1997, Van der Pauw et al. 2000, Nagano et al. 2004, 2006, Lossdorfer et al. 2007, Rodrigues et al. 2007). The presence of calcific deposition by EMD-treated PDL cells as an early marker of matrix mineralization was determined by alizarin red S and von Kossa staining. As expected, the calcium deposition was strongly enhanced by EMD, but this EMD-induced stimulation was partially diminished in the presence of IL-1β. Gene expression data for RUNX2 confirmed these results. Similar to our findings, Reseland et al. (2006) observed a stimulatory effect of EMD on factors involved in mineralization in human osteoblasts. However, although EMD increased the alkaline phosphatase activity as well as the expression of osteocalcin and COL1, RUNX2 was not affected, which is in contrast to our findings. Differences in cell type, duration of experiments, EMD concentration and origin of cells may be responsible for this discrepancy. Our findings suggest that IL-1β not only interferes with the EMD-induced effects on periodontal soft tissue regeneration but also inhibits the mineralization process under regenerative conditions.

Wound healing is characterized by inflammation, cell migration, and proliferation, followed by matrix deposition, and finally, tissue remodelling (Aukhil 2000) but whether and how much inflammation is needed for optimal tissue repair are as yet unknown (Eming et al. 2007, 2009). Furthermore, the effect of inflammation on the outcome of periodontal regenerative therapy has yet to be elucidated. Kobayashi et al. (1999) investigated the effect of several proinflammatory cytokines on the BMP2-stimulated alkaline phosphatase activity in PDL cells. They found that BMP2 exerts stimulatory effects on the osteoblastic differentiation of PDL cells, and additionally, that these effects of BMP2 are differentially regulated by inflammatory mediators, which points, at least in principle, at our findings. Additional evidence for inhibitory effects of inflammation on the regenerative capacity of tissues comes from studies in skin wound healing (Eming et al. 2007, 2009). For example, reduced systemic levels of oestrogen in ovariectomized mice are associated with excessive inflammation and impaired healing rate as well as scarring (Ashcroft et al. 1997, 2003). Furthermore, although wound healing of adult skin is characterized by lack of regeneration, healing of embryonic skin is regenerative (Redd et al. 2004). Important differences between scar-free healing in embryonic wounds and scar-forming healing in adult wounds include the inflammatory response, which in embryonic wounds consist of lower numbers of less differentiated inflammatory cells (Ferguson & O'Kane 2004). Furthermore, the growth factor profile in embryonic wounds differs qualitatively and quantitatively from that in scar-forming adult wounds. Embryonic wounds have lower levels of TGF-β1, TGF-β2, and platelet-derived growth factor but higher levels of TGF-β3 (Ferguson & O'Kane 2004). In rats, exogenous addition of TGF-β3 to cutaneous wounds has resulted in reduced or absent scarring (Shah et al. 1995), indicating that TGF-β3 may be a critical molecule for scar-free healing. TGF-β3 has also led to scar prevention and reduction in clinical trials (Occleston et al. 2008). However, timing of therapeutic intervention with growth factors, their antibodies or anti-inflammatory molecules to induce or promote regenerative healing may be critical (Lu et al. 2005). Both repair and regeneration can occur within the same animal and tissue, but by subtly altering the ratio of proinflammatory molecules and/or growth factors, the outcome of healing seems to be decisively affected (Ferguson & O'Kane 2004). Future studies should provide more mechanistic insight into the molecular and cellular processes by which inflammation can delay healing and impair its quality, because a better understanding of the underlying mechanisms may result in more powerful regenerative therapeutic strategies.

Interestingly, Myhre et al. (2006) have reported that EMD attenuates the release of tumour necrosis factor α and IL-8 from human blood cells challenged with lipopolysaccharide or peptidoglycan, indicating that EMD has anti-inflammatory properties. Similarly, EMD inhibited the lipopolysaccharide-stimulated tumour necrosis factor α production in rat monocytes (Sato et al. 2008). Therefore, it is conceivable that the beneficial effects of EMD on regenerative healing may be enhanced by downregulation of inflammatory mediators, i.e. creating an environment, which helps to maintain the full regenerative capacity of periodontal cells.

Periodontal inflammation is a highly complex process, which is difficult to simulate in vitro. In order to mimic an inflammatory environment in the present study, cells were exposed to IL-1β. However, other molecules are also upregulated at inflamed periodontal sites and it has yet to be determined how they, alone or in combination, affect the response of PDL cells to EMD.

Taken together, our study showed that EMD stimulated significantly migration, proliferation, and adhesion of PDL cells as well as matrix synthesis

and mineralization, and that the beneficial effects of EMD on these PDL cell functions were significantly reduced in an inflammatory environment. Within the limits of this in vitro study, we conclude that an efficient anti-infectious and anti-inflammatory periodontal treatment may be critical before the application of EMD.

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Clinical Relevance

Scientific rationale for the study: Most studies have investigated the effects of EMD under optimal conditions. Whether the actions of EMD on periodontal regeneration are affected by inflammation is as yet unknown.

Principal findings: The beneficial effects of EMD on PDL cell migration, proliferation, adhesion, matrix synthesis, and osteogenic differentiation were significantly reduced under simulated inflammatory conditions.
Practical implications: An efficient anti-infectious and anti-inflammatory periodontal treatment before the application of EMD may be critical in order to ensure the full regenerative capacity of the PDL tissue.