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Effects of enamel matrix derivative and transforming growth factor-β1 on human periodontal ligament fibroblasts

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Abstract

Aim: The objective of this study was to evaluate the effects of enamel matrix derivative (EMD), transforming growth factor-β1 (TGF-β1), and a combination of both factors (EMD+TGF-β1) on periodontal ligament (PDL) fibroblasts.

Material and methods: Human PDL fibroblasts were obtained from three adult patients with a clinically healthy periodontium, using the explant technique. The effects of EMD, TGF-β1, or a combination of both were analysed on PDL cell proliferation, adhesion, wound healing, and total protein synthesis, and on alkaline phosphatase (ALP) activity and bone-like nodule formation.

Results: Treatment with EMD for 4, 7, and 10 days increased cell proliferation significantly compared with the negative control ($p<0.05$). At day 10, EMD and EMD+TGF-β1 showed a higher cell proliferation compared with TGF-β1 ($p<0.01$). Cell adhesion was significantly up-regulated by TGF-β1 compared with EMD and EMD+TGF-β1 ($p<0.01$). EMD enhanced in vitro wound healing of PDL cells compared with the other treatments. Total protein synthesis was significantly increased in PDL cells cultured with EMD compared with PDL cells treated with TGF-β1 or EMD+TGF-β1 ($p<0.05$). EMD induced ALP activity in PDL fibroblasts, which was associated with an increase of bone-like nodules.

Conclusion: These findings support the hypothesis that EMD and TGF-β1 may play an important role in periodontal regeneration. EMD induced PDL fibroblast proliferation and migration, total protein synthesis, ALP activity, and mineralization, while TGF-β1 increased cellular adhesion. However, the combination of both factors did not positively alter PDL fibroblast behaviour.

Key words: enamel matrix derivative; fibroblasts; growth factors; periodontal; periodontal ligament; TGF-β1

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Enamel matrix derivatives (EMD), derived from porcine enamel matrix proteins, have been used for periodontal regeneration with clinically satisfactory results. These proteins, produced by Hertwig's epithelial sheath, are known to play an important role in cementogenesis as well as in the development of the periodontal attachment apparatus (Ten Cate 1996, Hammarström 1997). Clinical investigations have demonstrated that application of EMD during periodontal treatment results in regeneration of the acellular cementum, significant clinical attachment gain, and radiographic bone filling (Brooks et al. 1995, Hammarström 1997, Heijl 1997, Heijl et al. 1997, Mellonig 1999).

In vitro studies have demonstrated that EMD treatment of periodontal ligament (PDL) fibroblasts stimulates proliferation, protein and collagen synthesis, and also induces mineralization (Gestrelius et al. 1997, Van der Pauw et al. 2000, Palioto et al. 2004). Palioto et al. (2004) demonstrated that EMD increases PDL fibroblast proliferation, but migration and

Conflict of interest and source of funding statement

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adhesion of these cells are unaffected. In addition, Lyngstadaas et al. (2001) suggested that EMD can enhance the synthesis of transforming growth factor-β (TGF-β) in PDL cells and that this secondary effect may, in part, explain some of the activities attributed to EMD.

The use of polypeptide growth factors, which function as potent biologic mediators regulating numerous activities of wound healing, has been suggested for the promotion of periodontal regeneration (Terranova & Wikesjo 1987, Lynch et al. 1989). The fundamental functions of growth factors as mitogens, chemoattractants, and in the stimulation of collagen synthesis have been relatively well characterized (Matsuda et al. 1992). Terranova & Wikesjo (1987) hypothesized that growth factors may be used to obtain biochemically mediated regeneration of the periodontium. One polypeptide growth factor of interest is TGF-β1, which has been reported to facilitate tissue repair and regeneration (Assoian et al. 1983, Terranova et al. 1989).

TGF-β1 is a multifunctional peptide that regulates various cellular activities, including proliferation, differentiation, and expression of extracellular matrix proteins (Kingsley 1994). Several studies have shown that TGF-β1 is a potent inhibitor of epithelial cell proliferation, while it enhances the proliferation of mesenchymal cells, such as fibroblasts and osteoblasts (Sporn et al. 1987, Crabb et al. 1990, Graves & Cochran 1991, Royce & Baum 1991). It has been reported that TGF-β1 alone or in combination with other growth factors accelerates various phases of wound healing (Lynch et al. 1991, Canalis 1992). TGF-β1 stimulates new granulation tissue formation through angiogenesis and collagen production by fibroblasts (Lynch et al. 1989, Dennison et al. 1994). Addition of TGF-β1 to gingival and periodontal ligament fibroblast cell cultures has been shown to enhance RNA and protein synthesis (Mailhot et al. 1995).

The combination of growth factors with EMD has the objective to enhance the positive effects of each one on periodontal regeneration and this combination has actually been little explored, but it is potentially viable and possibly interesting to understand the mechanism of how EMD acts. Palioto et al. (2004) showed that the combination of EMD with insulin-like growth factor-I (IGF-I) enhanced proliferation but did not promote additional effects in the adhesion, migration, expression, or production of type I collagen by PDL fibroblasts than those obtained with EMD alone. The aim of the present study was to evaluate the effects of EMD, TGF-β1, and their combination on proliferation, adhesion, migration, total protein synthesis, mineralization, and ALPase activity in human PDL fibroblasts.

Material and Methods

Cell culture

Human PDL fibroblasts were obtained from premolars or third molars extracted for orthodontic reasons from three adult patients with clinically healthy periodontium, using explant cultures as described previously by Somerman et al. (1989). Briefly, premolars or third molars were extracted, washed twice with saline, and the PDL fragments from the middle third of the root were cureted. The fragments were washed and cultured in Dulbecco's-modified Eagle medium (DMEM – Gibco BRL, Gaithersburg, MD, USA) containing 10% foetal bovine serum (FBS – Gibco BRL), 50 μg/ml vancomicin (Acros, Fair Lawn, NJ, USA), and 20 μg/ml ampicillin (USB Corporation, Cleveland, OH, USA) at 37°C in a 5% CO_2 air atmosphere. When cells growing out from explants reached confluence, they were trypsinized with 0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA – Gibco BRL) in phosphate-buffered saline (PBS – Gibco) for the second culture. All experiments were performed using cells between the third and the eighth passages. All patients were informed about the study's purpose before they consented to participate. The Ethics Committee on Research of the University of São Paulo approved the protocol.

Treatments

Emdogain gel (EMD – Biora, Malmo, Sweden) was dissolved in acidic water, pH 5.9, whereas TGF-β1 (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in acetonitrilo plus trifluoracetic acid (Sigma Chemical Co.). Both solutions were aliquoted and stored at − 70°C. Based on our previous studies, treatment with EMD and TGF-β1 was performed at concentrations of 100 and 5 ng/ml, respectively (Martelli-Junior et al. 2003, Palioto et al. 2004). Five experimental conditions were established: (1) media containing 10% FBS (positive control); (2) media with 2% charcoal-treated (CT) FBS (negative control); (3) 100 μg/ml EMD in 2% CT-FBS media; (4) 5 ng/ml TGF-β1 in 2% CT-FBS media; and a (5) combination of 100 μg/ml EMD and 5 ng/ml TGF-β1 in 2% CT-FBS media.

Cell growth assay

The cell growth assay was performed using a modified method of Coletta et al. (1998). PDL fibroblasts were plated in a 24-well culture plate (Corning Inc., NY, USA) at a density of 30,000 cells/well in 2 ml of DMEM containing 10% FBS, 50 μg/ml vancomicin (Acros), and 20 μg/ml ampicillin (USB Corporation). The cells were allowed to attach and spread for 24 h, and then washed with PBS and cultured in serum-free DMEM for an additional 24 h. After treatments with the five experimental conditions for 1, 4, 7, and 10 days, cells were enzymatically harvested with 0.05% trypsin and 0.02% EDTA in PBS. Aliquots of these solutions were incubated for 5 min. with the same volume of trypan blue and directly counted in a haemocytometer (Fisher Scientific, Pittsburgh, PA, USA) to determine number of cells $\times 10^4$/well. For each time point, total cell number was determined.

Bromodeoxyuridine-labelling (BrdU) index

PDL fibroblasts were plated on 8-well glass culture chamber slides (Nunc International, Naperville, IL, USA) at a density of 30,000 cells/well in 500 μl of DMEM containing 10% FBS, 50 μg/ml vancomicin (Acros), and 20 μg/ml ampicillin (USB Corporation), and were incubated at 37°C and 5% CO_2. Following 24 h of serum starvation, cells were exposed to the five experimental culture conditions for 24 h. After treatment, cells were incubated with BrdU (diluted 1:1000) for 1 h under the same conditions, washed in PBS, and fixed in 70% ethanol for 15 min. BrdU incorporation in proliferating cells was revealed using immunohistochemistry (Amershan Pharmacia Biotech Inc., Piscataway, NJ, USA). Briefly, the anti-5-bromo-2′-deoxyuridine monoclonal antibody, diluted 1:100 in nuclease with deionized water, was added to the wells and incubated for 1 h. The wells were then washed three

times with 500 μl of PBS and the peroxidase anti-mouse IgG2a (15:1000) were added to the wells and incubated for more than 1 h. After another washing step, the reaction was developed with 0.6 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) containing 1% of H_2O_2 and 1% of DMSO for 5 min. at 37°C. Later, the cells were stained with Crazzi haematoxylin and examined under optical microscopy. The BrdU-labelling index, expressed as the percentage of cells labelled with BrdU, was determined by counting 1500 cells using an image analysis system (Kontron 400, Zeiss, Eching bei Munich, Germany).

Cell adhesion assay

A cell adhesion assay was performed using the methods of Hebert et al. (2001) with some modifications. Briefly, 24-well culture plates were sensitized with 2 ml of PBS containing EMD, TGF-β1, or EMD+TGF-β1 at the same concentrations described above for 16 h at 4°C, and were subsequently washed three times with PBS. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA–Sigma) in PBS for 2 h at room temperature. Cells were released from tissue culture flasks with 0.05% trypsin and 0.02% EDTA, washed with the adhesion medium (DMEM containing 2% CT-FBS and 3% BSA), and plated onto 24-well plates with a density of 30,000 cells in 500 μl of adhesion medium plus factors. Incubation was performed for 2 h at 37°C in 5% CO_2. Following incubation, wells were gently rinsed with 1 ml PBS to remove unattached cells. At this time, photographs were taken to document cell spreading, using an inverted light microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (Canon EOS Digital Rebel Camera, 6.3 Megapixel CMOS sensor, Canon USA Inc., Lake Success, NY, USA). Attached cells were harvested with trypsin, and counted in a haemocytometer. Untreated cells in uncoated plates were used as negative control. Data are expressed as the percentage of attached cells compared with control.

In vitro wound healing model

The effects of EMD, TGF-β1, or their combination on PDL cell migration were analysed by an in vitro wound-healing model (Hoang et al. 2000). A confluent monolayer of PDL cells was incubated for 24 h in 0.2% FBS. After this, in vitro wounds were created by removing the cellular and extracellular contents across a 3 mm wide area of the well. The wound was made in the central portion of the well and extended to the diameter of the well, and long linear marks were made along wound edges to mark the starting point of cell migration. The wells were washed three times with PBS before incubation under the same conditions as described above. Following 2 and 6 days of incubation, cells were fixed with 70% ethanol for 16 h, and subsequently photographed using an inverted light microscope equipped with a digital camera. Wound repopulation was qualitatively analysed by two trained and blinded evaluators.

Total protein synthesis

PDL fibroblasts were plated in 24-well culture plates at a density of 20,000 cells/well in 2 ml of DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone (USB Corporation), 7 mM β-glycerophosphate (USB Corporation), 5 μg/ml ascorbic acid (USB Corporation), 50 μg/ml vancomicin (Acros), and 20 μg/ml ampicillin (USB Corporation) at 37°C in a humidified atmosphere with 5% CO_2. These culture conditions favour the development of the osteoblast phenotype (Coelho & Fernandes 2000, Rosa & Beloti 2003). Following serum starvation, cells were exposed to the five experimental culture conditions described previously with differentiation medium for 14 days. Media were changed and supplemented every 3 or 4 days. The total protein content was determined using a modification of the Lowry method (Lowry et al. 1951). Briefly, proteins were extracted from each well with 0.1% sodium lauryl sulphate (Sigma) for 30 min., resulting in a lysate of the cells, and mixed 1:1 with Lowry solution (Sigma) for 20 min. at room temperature. The resulting solution was diluted in Folin and Ciocalteau's phenol reagent (Sigma) for 30 min. at room temperature. Absorbance was measured at 680 nm using a spectrophotometer (Cecil CE3021, Cambridge, UK). The total protein content was calculated from a standard curve and expressed as μg/ml.

Alkaline phosphatase (ALP) activity

The same PDL fibroblasts plated to assess total protein synthesis were also used for ALP activity, and the lysates obtained with the addition of 0.1% sodium lauryl sulphate (Sigma) solution were used. ALP activity was measured as the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica, MG, Brazil). Briefly, 50 μl thymolphthalein monophosphate was mixed with 0.5 ml 0.3 M diethanolamine buffer, pH 10.1, and left for 2 min. at 37°C. The solution was then added to 50 μl of the lysates obtained from each well for 10 min. at 37°C. For colour development, 2 ml 0.09 M Na_2CO_3 and 0.25 M NaOH were added. After 30 min., absorbance was measured at 590 nm and ALP activity was determined from a standard curve using thymolphthalein to yield a range from 0.012 to 0.4 μmol thymolphthalein/h/ml. Data were expressed as ALP activity normalized for total protein content at 14 days.

Mineralized bone-like nodule formation

PDL fibroblasts were plated in 24-well culture plates at a density of 20,000 cells/well in 2 ml of DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone (USB Corporation), 7 mM β-glycerophosphate (USB Corporation), 5 μg/ml ascorbic acid (USB Corporation), 50 μg/ml vancomicin (Acros), and 20 μg/ml ampicillin (USB Corporation) at 37°C in a humidified atmosphere with 5% CO_2. Following serum starvation, cells were exposed to the five experimental culture conditions described previously with differentiation medium for 21 days. Media were changed and supplemented every 3 or 4 days. At day 21, cultures were washed in PBS and fixed with 10% formaldehyde in PBS, pH 7.2, for 16 h at 4°C. The samples were then dehydrated in a graded series of alcohol and stained with 2% Alizarin red S (Sigma), pH 4.2, for 8 min. at room temperature. The same two trained and blinded evaluators, using an inverted light microscope ($\times$ 10 objective – Carl Zeiss), with a Canon camera attached, analysed qualitatively the bone-like nodule formation, observing the frequency and the appearance (dense or diffuse) of the formed nodules.

Statistical analysis

All experiments were performed at least twice in triplicate for each cell line. Data represent mean and standard deviation (SD) of three cell lines, and the non-parametric Kruskal–Wallis test for

independent samples was used for statistical analyses. If the result of the Kruskal–Wallis test was significant ($p<0.05$), Fisher's test for multiple comparisons, computed on ranks rather than data, was performed (Conover 1980).

Results

Effect of EMD, TGF-β1, or both on cell proliferation

The effect of EMD, TGF-β1, and the combination of both on PDL fibroblast proliferation was assessed by direct counting of cell number and BrdU incorporation into DNA. EMD treatment significantly increased the proliferation rate of PDL cells at 4, 7, and 10 days when compared with the negative control (Fig. 1 and Table 1; $p<0.05$ to 4 days, $p<0.01$ to 7 days, $p<0.001$ to 10 days). On day 10, proliferation of PDL fibroblasts treated with EMD was also statistically higher than with TGF-β1 ($p<0.001$). Although with a small difference, on day 10, treatment with EMD+TGF-β1 induced statistically significant PDL proliferation rates compared with TGF-β1 alone (Fig. 1; $p<0.05$). For all treatment modalities, proliferation was significantly lower than the positive control at all time points ($p<0.05$), except on day 10, when proliferation of cells on EMD was higher than the positive control.

Nuclear immunoreactivity for BrdU was clearly and easily identified in PDL cells under all treatments. Nuclei with a clear brown colour, regardless of the intensity of staining, were interpreted as positive. BrdU-labelling indices after specific treatment of PDL cells are shown in Fig. 2. All treatments affected PDL proliferation at the first 24 h of the experiment, even though without statistical significance.

Effect of EMD, TGF-β1, or both on cell adhesion assay

EMD and a combination of EMD and TGF-β1 statistically inhibited cell adhesion compared with control and TGF-β1 alone (Fig. 3; $p<0.001$). Cells plated with EMD or EMD+TGF-β1 were not spread out evenly, forming clusters of largely rounded cells, whereas cells plated with control and TGF-β1 alone showed proper attachment with a typical fibroblastic morphology (Fig. 4).

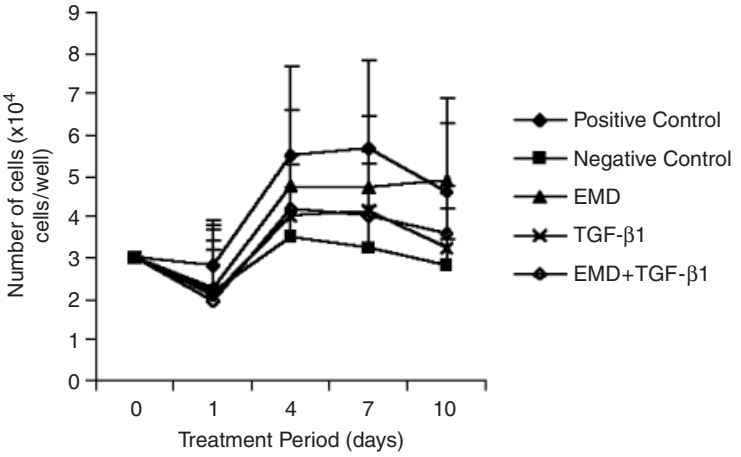

Fig 1. Enamel matrix derivative (EMD) induces proliferation of periodontal ligament (PDL) fibroblasts. Effect of EMD, transforming growth factor-β1 and the combination of both factors on cell growth. Values are expressed as the mean ± SD of three cell lines in two independent experiments, each conducted in triplicate. From day 4 to 10, the growth rates of PDL cells treated with EMD were higher than control.

Table 1. Statistical analysis for Fig. 1

	Day 1			Day 4		
	EMD	TGF-β1	EMD+TGF-β1	EMD	TGF-β1	EMD+TGF-β1
Positive control	NS	NS	*	NS	*	*
Negative control	NS	NS	NS	*	NS	NS

	Day 7			Day 10		
	EMD	TGF-β1	EMD+TGF-β1	EMD	TGF-β1	EMD+TGF-β1
Positive control	NS	†	*	NS	*	NS
Negative control	†	NS	NS	‡	NS	*
TGF-β1	NS	NS	NS	‡	NS	*

*$p<0.05$; †$p<0.01$; ‡$p<0.001$.
NS, not significant; EMD, enamel matrix derivative; TGF-β1, transforming growth factor-β1.

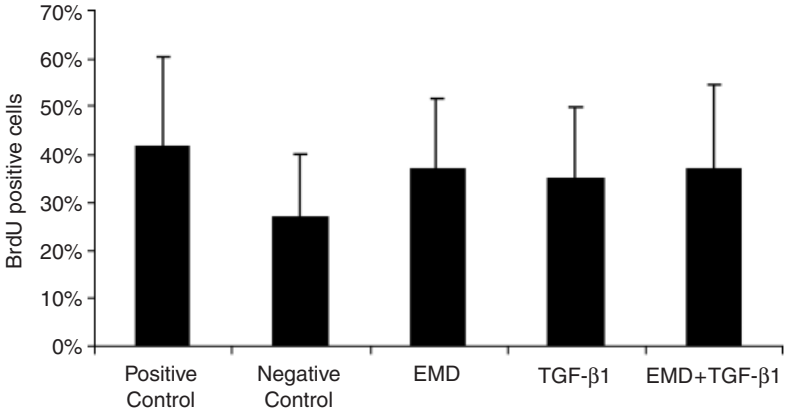

Fig 2. BrdU-labelling indices of periodontal ligament (PDL) cells under treatment with enamel matrix derivative, transforming growth factor-β1 (TGF-β1), and a combination of both factors. Data correspond to the mean percentage of positive cells of three strains of PDL fibroblasts.

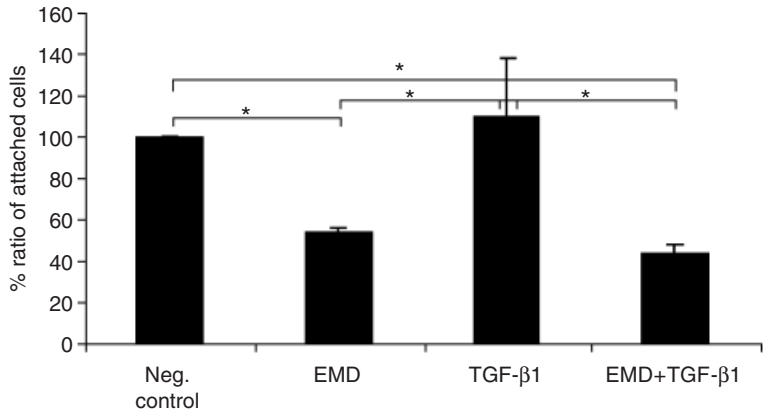

Fig 3. Effect of enamel matrix derivative (EMD), transforming growth factor-β1 (TGF-β1), and both on periodontal ligament (PDL) fibroblast adhesion. The results are expressed as a percentage ratio of attached cells compared with bovine serum albumin-negative control. EMD and EMD+TGF-β1 significantly inhibited PDL fibroblast adhesion. $^{*}p<0.001$.

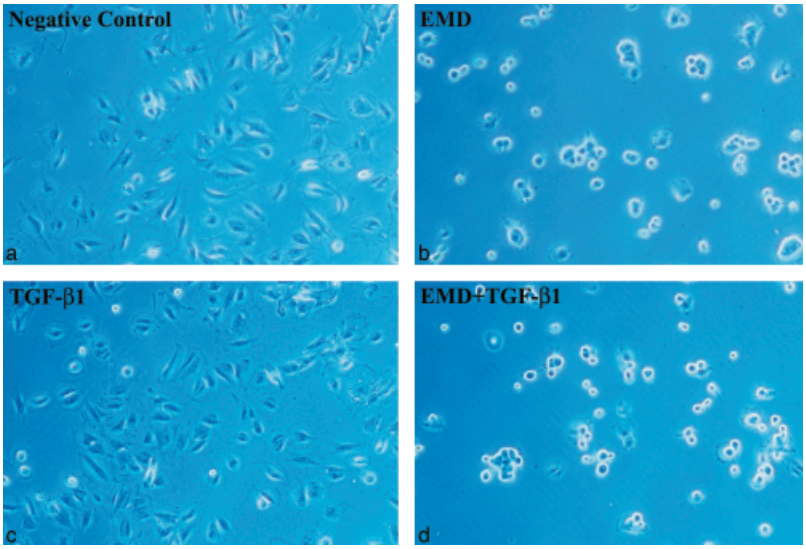

Fig 4. Representative inverted light microscope view of one of the cell lines of this study after a 2-h adhesion on plates coated with bovine serum albumin negative control (a), 100 μg/ml enamel matrix derivative (EMD) (b), 5 ng/ml transforming growth factor-β1 (TGF-β1) (c), and 100 μg/ml EMD plus 5 ng/ml TGF-β1 (d). Periodontal ligament cells plated on EMD or EMD+TGF-β1-coated surfaces did not spread out properly, forming cell clamps.

Effect of EMD, TGF-β1, or both on an in vitro wound model

Migration of PDL fibroblasts treated with EMD, TGF-β1, and a combination of these two factors was qualitatively analysed in a wound-healing model (Hoang et al. 2000). EMD accelerated wound filling compared with negative control and the other two treatments at 2 and 6 days post-wounding. In the analysed periods, EMD+TGF-β1 demonstrated higher wound filling when compared with TGF-β1 (Fig. 5).

Effect of EMD, TGF-β1, or both on total protein synthesis, ALP activity, and bone-like nodule formation

Total protein synthesis was affected by all treatments. PDL cells with EMD treatment produced statistically more protein than with other treatments ($p<0.05$). TGF-β1 alone or in combination with EMD increased protein production by PDL fibroblasts compared with negative control. Although not significantly different ($p>0.05$), TGF-β1 alone induced protein synthesis by PDL cells compared with EMD+TGF-β1 (Fig. 6).

ALP activity by PDL cells was positively affected by EMD treatment compared with negative control, TGF-β1, and EMD+TGF-β1 ($p<0.01$). Although the positive control expressed more ALP activity than the EMD group, this activity was not statistically significant (Fig. 7). After 21 days, it was observed that EMD moderately induced cellular differentiation. Dense areas with bone-like nodule formation and diffuse areas with initial nodule formation were consistently observed in PDL cultures treated with EMD. In TGF-β1 and EMD+TGF-β1 treatments, areas with diffuse initial mineralization were rarely observed and dense mineralized nodules were not observed (Fig. 8).

Discussion

Periodontal regeneration is dependent on a sequence of associated events including cellular proliferation, migration, and attachment to components of the extracellular matrix as well as organic matrix synthesis and mineralization (Rincon et al. 2003). EMD was introduced as an adjuvant to periodontal treatment due to its ability to control some of the critical events associated with periodontal regeneration, such as cementogenesis and development of the periodontal attachment apparatus. Despite extensive studies, the exact mechanism by which EMD induces periodontal regeneration is unknown, and clinical and cell-culture studies are controversial regarding the differences between in vitro and in vivo results in periodontal regeneration (Gestrelius et al. 1997, Davenport et al. 2003, Gurpinar & Onur 2003, Okubo et al. 2003). Furthermore, recent studies in vitro have combined growth factors, such as IGF, with EMD to improve its activity, resulting in a positive enhancement of cell events of periodontal regeneration, such as cell proliferation and protein production (Lyngstadaas et al. 2001, Palioto et al. 2004). As TGF-β1 can regulate various cellular activities including growth, differentiation, and expression of extracellular matrix proteins (Assoian et al. 1983, Streuli et al. 1993, Ivanovski et al. 2001), we sought to determine whether the combination of EMD and TGF-β1 provided additional advantages for the cellular events associated with periodontal regeneration.

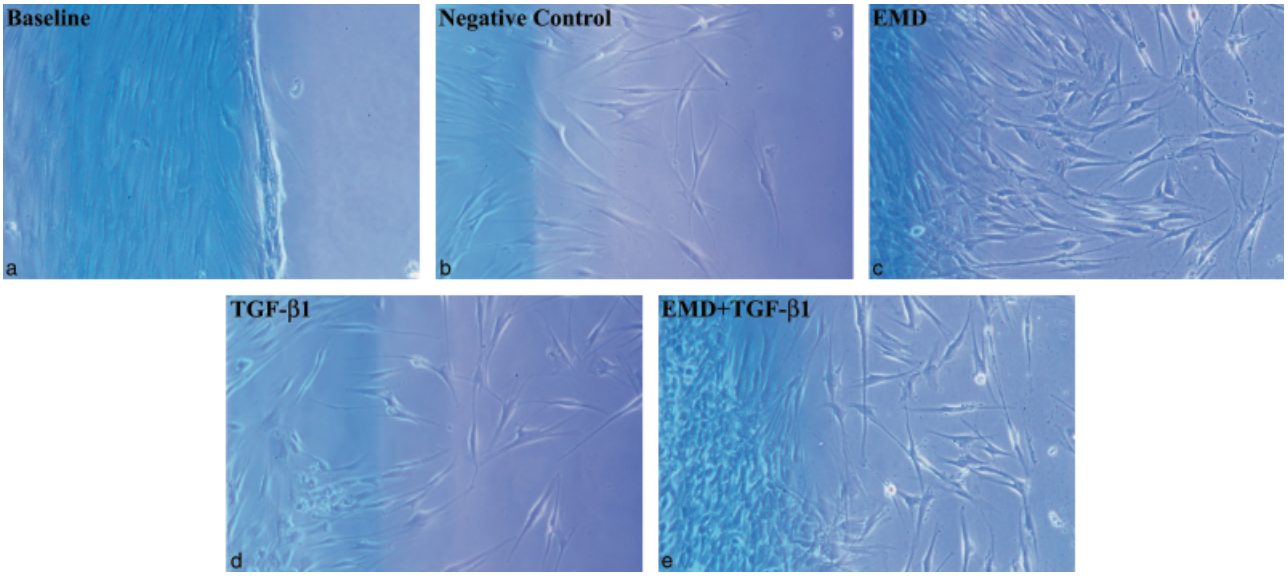

Fig 5. Enamel matrix derivative (EMD) and EMD plus transforming growth factor-β1 (TGF-β1) enhanced periodontal ligament fibroblast migration associated with wound repair. (a) View of the baseline creating a wound scenario and (b) cells cultured in media containing 2% CT-FBS used as control. Representative inverted light microscope view of the one of the cell lines of this study treated for 2 days with 100 μg/ml EMD (c), 5 ng/ml TGF-β1 (d), and 100 μg/ml EMD plus 5 ng/ml TGF-β1 (e).

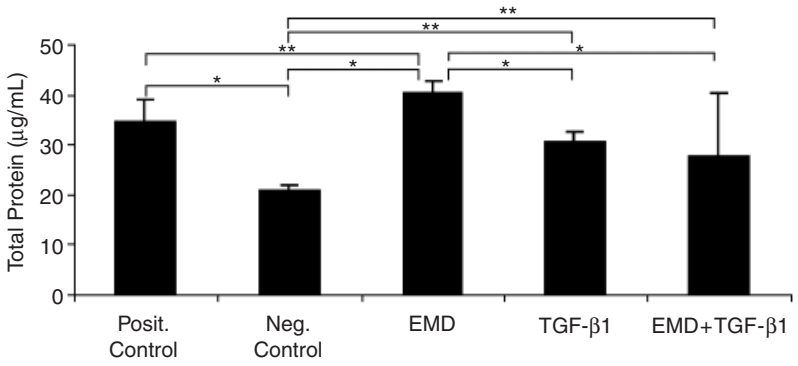

Fig 6. Treatment with enamel matrix derivative (EMD) stimulated total protein synthesis by periodontal ligament fibroblasts. Values are expressed as mean $\pm$ standard deviation of three cell lines performed in two independent experiments in triplicate. $^{*}p<0.001$, $^{**}p<0.05$.

The results presented here showed that the treatment with EMD and TGF-β1 affect human PDL fibroblasts, but a combination of both factors did not induce any effect for the two other factors used alone. EMD stimulated proliferation, migration, total protein synthesis, ALP activity, and bone-like nodule formation. Interestingly, the combination of both factors showed that the influence of EMD was greater than TGF-β1, as demonstrated by the fact that most of the results followed the same trend of EMD treatment alone.

In accordance with previous studies (Gestrelius et al. 1997, Palioto et al. 2004), we found that EMD promoted proliferation of PDL in a time-dependent manner. EMD significantly increased the rate of proliferation when compared with negative control from day 4 to 10 and compared with TGF-β1 at day 10. Interestingly, PDL fibroblasts responded to the proliferative stimuli of EMD at a late stage, as revealed by the BrdU incorporation assay. On the other hand, TGF-β1 did not alter PDL fibroblast proliferation. Matsuda et al. (1992) also reported that at a concentration of 10 ng/ml TGF-β1 did not improve the proliferation rate of PDL cells. This is conflicting with previous reports (Oates et al. 1993, Dennison et al. 1994). Oates et al. (1993) demonstrated that TGF-β1 significantly increased the mitogenic activity of PDL cells. Dennison et al. (1994) found that TGF-β1 stimulated the proliferation of PDL cells, and that proliferative response of PDL cells to platelet-derived growth factor was enhanced by TGF-β1. It is well established that TGF-β1 acts in a dose-dependent manner, and its mitogenic effect is variable depending on the drug concentration and culture conditions (Matsuda et al. 1992).

Although Gestrelius et al. (1997) demonstrated that EMD did not contain any growth factors, others have suggested that EMD acts as a natural and efficient drug-delivery system for exogenous TGF-β1 and other similar factors (Kawase et al. 2002). The stimuli of EMD on cells may not be the direct effect of enamel matrix but due more to the presence of various types of growth factors, which are contained in EMD itself and/or medium conditioned by EMD-treated cells that became rich in endogenous cytokines (Van der Pauw et al. 2000, Okubo et al. 2003). It has also been reported that EMD has TGF-β-like activity on both oral epithelial and fibroblastic cell cultures (Kawase et al. 2002). In the present study, we found opposite features when the cells were stimulated by exogenous TGF-β1 or by EMD.

Our adhesion assay showed that EMD and the combination of EMD and TGF-β1 significantly decreased PDL fibroblast attachment, which is in agreement

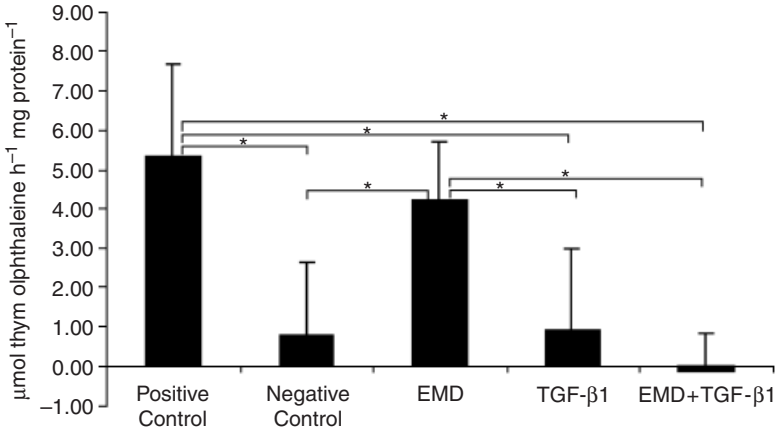

Fig 7. Effect of enamel matrix derivative (EMD), transforming growth factor-β1 (TGF-β1), and EMD plus TGF-β1 on alkaline phosphatase activity of periodontal ligament fibroblasts. The results are expressed as μmol thymolphthaleine/h/mg protein, and represent mean standard deviation of three cell lines in triplicate. $^{*}p<0.01$.

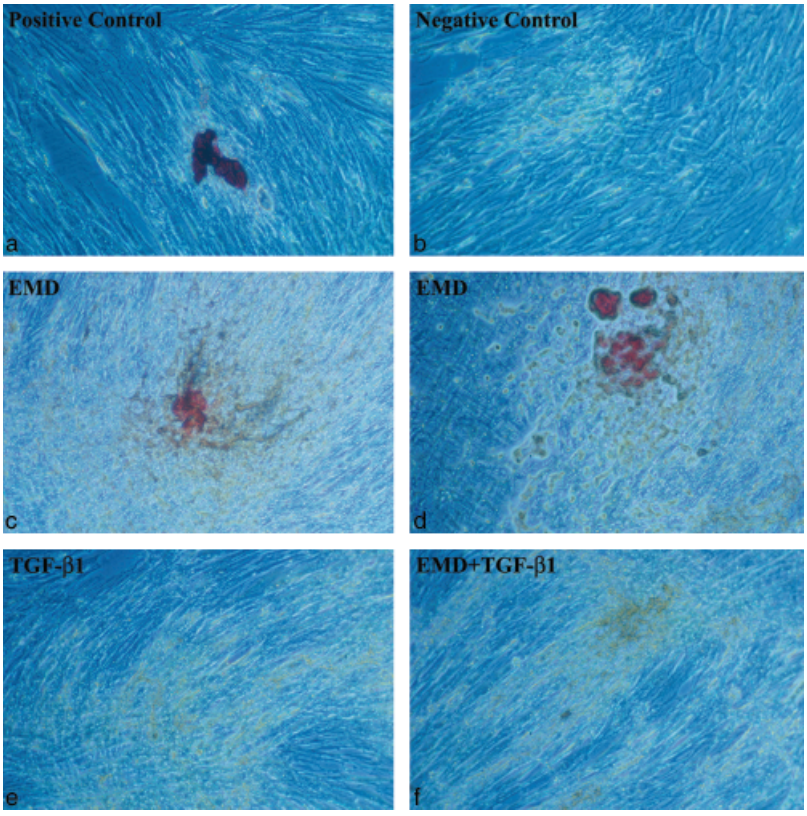

Fig 8. Representative alizarin red stained extracellular mineral nodules formed by periodontal ligament cells under treatment with Dulbecco's-modified Eagle medium (DMEM) and 10% foetal bovine serum (FBS) as positive control (a), DMEM and 2% CT-FBS as negative control (b), 100 μg/ml enamel matrix derivative (EMD) (c, d), 5 ng/ml transforming growth factor-β1 (TGF-β1) (e), and 100 μg/ml EMD plus 5 ng/ml TGF-β1 (f). Note in c that EMD induced a diffuse bone-like nodule formation and in d more compact bone-like nodules (original magnification $\times$ 10).

with previous studies (Gestrelius et al. 1997, Palioto et al. 2004). Our results also revealed that PDL cells treated with EMD and EMD+TGF-β1 were not properly spread out, and formed clampers of largely rounded cells, which may be due to the non-soluble characteristics of EMD proteins. Likewise, Van der Pauw et al. (2000) showed that compared with collagen as a substratum, EMD had an inhibitory influence on attachment and spreading of PDL cells. Lyngstadaas et al. (2001) found a five-fold increase in cell adhesion on plates coated with EMD. These conflicting results may be due to differences in the coating procedures or to the higher concentration (500 μg/ml) used by these authors. On the other hand, TGF-β1 induced PDL fibroblast attachment. Cells plated on TGF-β1-coated surfaces were robust and with a denditric appearance, suggesting that TGF-β1 induces the production of extracellular matrix proteins and its receptors, enhancing cell adhesion (Ivanovski et al. 2001).

Wound healing is a complex process involving cell migration, cell attachment to various components of the extracellular matrix, and cell proliferation (Rincon et al. 2003). Many of these processes are controlled by cytokines and growth factors. In our results, EMD and TGF-β1 enhanced migration associated with wound filling of PDL cells compared with other treatments. A previous study using a different wound-healing model has described a similar effect of EMD on wound closure responses of periodontal ligament fibroblasts (Rincon et al. 2003). Hoang et al. (2000), using the same in vitro wound model that we used, showed that when PDL, gingival fibroblasts, and MG-63 cells were exposed to EMD, there was enhanced wound fill for all cells compared with untreated conditions, mainly at early times.

The present study also revealed that EMD induced total protein synthesis, ALP activity, and bone-like nodule formation in PDL cultures. Furthermore, EMD induced mineralization when cultured in differentiation media, and in the media without ascorbic acid and β-glycerophosphate (Nagano et al. 2004). Addition of TGF-β1 to EMD culture media did not substantially affect EMD effects on those phenotypes. According to Haase & Bartold (2001), EMD appears to stimulate matrix synthesis in vitro through modulation of hylaruonan and proteoglycan synthesis. On the other hand, Gestrelius et al. (1997) demonstrated that the effect of EMD on PDL fibroblast total protein synthesis is partially dependent on the up-regulation on collagen production.

Studies have demonstrated that ALP activity and bone-like nodule formation were significantly enhanced in the

presence of EMD in human PDL cells (Gestrelius et al. 1997, Van der Pauw et al. 2000, Nagano et al. 2004). Furthermore, EMD upregulated gene expression of tissue specific differentiation markers for osteoblasts, including osteopontin, osteocalcin, and bone sialoprotein (Kawase et al. 2002). In contrast, Okubo et al. (2003) showed that EMD might not have an appreciable effect on osteoblastic differentiation in PDL cells. Similarly, Hakki et al. (2001) and Tokiyasu et al. (2000) showed that EMD totally blocked follicle cell and cementoblast-mediated mineral nodule formation in vitro. Thus, according to Okubo et al. (2003), the in vitro effect of EMD on PDL fibroblast differentiation into osteoblastic-like cells is controversial. This may be because human periodontal ligament fibroblasts differ in phenotype depending on the method used for isolation, the condition of the donor, i.e. age, and source of the cells, i.e. alveolar or ligament brought with the root surface during extraction.

Conclusion

The present study showed that EMD increased PDL fibroblast proliferation, migration, total protein synthesis, ALP activity, and mineralization, while TGF-β1 increased cellular adhesion. The combination of both factors did not positively influence PDL fibroblasts in the analysed features of this study. The results of the combination showed that the influence of EMD was greater than TGF-β1 because most of the results followed the same trend of treatment with EMD alone.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. EMD induces proliferation of PDL fibroblasts. Effect of EMD, TGF-β1, and a combination of both factors on cell growth. Values are expressed as the mean $\pm$ SD of 3 cell lines in two independent experiments, each conducted in triplicate. From day 4 to 10, the growth rates of PDL cells treated with EMD were higher than control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

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Clinical Relevance

Scientific rationale for the study: EMD plays an important role in the development of the periodontal attachment apparatus. The combination of growth factors with EMD has the objective to enhance the positive effects of each one on periodontal regeneration and this combination has been little explored, but it is potentially viable and possibly interesting to understand the mechanism of how EMD acts. Therefore, we sought to determine if the combination of these two factors would make PDL cells work in their maximum regenerative capacity.

Principal findings: The results showed that EMD increased PDL fibroblast proliferation, migration, total protein synthesis, ALP activity, and mineralization, while TGF-β1 increased cellular adhesion.

Practical implications: Clinical investigations demonstrated that the use of EMD during periodontal surgery results in a significant gain in clinical attachment and radiographic bone fill (Heijl et al. 1997, Mellonig 1999), and has the potential to induce regeneration in animal models (Hammarström et al. 1997). Therefore, the results suggest a positive action of EMD on PDL cells.