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ORIGINAL ARTICLE

Frank Schwarz · Daniel Rothamel · Monika Herten · Anton Sculean · Werner Scherbaum · Jürgen Becker

Effect of enamel matrix protein derivative on the attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on titanium implants

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Abstract The purpose of the present study was to investigate the effects of an enamel matrix protein derivative (EMD) on attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on titanium implants. A total of 220 sand-blasted and acid-etched (SLA) titanium discs were placed into 24-well culture plates. Before cell inoculation, McCoy's 5A medium (MCM) containing EMD at 25 μg/ml, 50 μg/ml, 100 μg/ml, and 200 μg/ml was added, and the culture plates were incubated for 30 min. As control, MCM alone was used. Human osteoblast-like cells ($SaOs_2$) (2×10^4 cells, fourth passage) were suspended in MCM containing 1% penicillin/streptomycin and 10% fetal bovine serum and then inoculated into the well chambers. The medium was changed after 3 days without the addition of EMD. At days 1, 3, and 6, DNA content of the cells was assessed using the CyQuant cell proliferation assay kit, and mitochondrial activity of the cells was measured using a CellTiter-Glo luminescent cell viability assay. The presence of EMD on the titanium discs at days 1 and 6 was evaluated using immunofluorescence stain (IFS) by means of polyclonal antibodies against amelogenin. Additionally, cell morphology was investigated using scanning electron microscopy. Enamel matrix derivative at 25 μg/ml, 50 μg/ml, 100 μg/ml, and 200 μg/ml demonstrated similar increases in cell proliferation as the control medium at days 3 and 6 ($P>0.05$ between groups, respectively). Proliferation, however, appeared to be ameliorated with increasing EMD concentrations. At 25 μg/ml and 50 μg/ml, EMD also demonstrated an increase in cell viability similar to the control medium at days 3 and 6 ($P>0.05$ between groups, respectively), while EMD at 100 μg/ml and 200 μg/ml resulted in statistically significant higher increase in cell viability than in the control medium at day 6 ($P<0.001$ between groups, respectively). In all test groups, IFS at day 6 was markedly lower than at day 1. Scanning electron microscopy revealed comparable cell morphology in all groups. Within the limits of the present study, it was concluded that EMD enhanced cell proliferation and viability of human $SaOs_2$ osteoblasts on SLA titanium implants in a concentration-dependent manner.

Keywords Enamel matrix protein derivative · Human $SaOs_2$ osteoblasts · Proliferation · Titanium implants · Viability

F. Schwarz (✉) · D. Rothamel · M. Herten · J. Becker
Department of Oral Surgery, Heinrich Heine University,
Westdeutsche Kieferklinik,
Moorenstrasse 5, 40225 Düsseldorf, Germany
e-mail: info@frank-schwarz.de
Tel.: +49-211-8118149
Fax: +49-211-1713542

A. Sculean
Section of Periodontology,
Department of Conservative Dentistry and Periodontology,
Johannes Gutenberg University,
55101 Mainz, Germany

W. Scherbaum
German Diabetes Center,
Heinrich Heine University,
Düsseldorf, Germany

Introduction

Enamel matrix proteins secreted by Hertwig's epithelial sheath are known to play important biological roles in cementogenesis and development of the periodontal attachment apparatus [16, 30]. The enamel matrix protein isolated from developing porcine teeth is called enamel matrix protein derivative (EMD) and also referred to as Emdogain (Biora, Malmö, Sweden), that constitutes up to 90% of amelogenins [12]. Histologic findings from animals [12] and humans [19, 26, 33] have shown that the application of EMD onto a debrided root surface may also induce the formation of cementum and collagenous fibers.

Findings from controlled clinical trials provide clear evidence that treatment of intrabony defects with EMD may result in clinical outcomes comparable to those following guided tissue regeneration [27, 29]. Furthermore, clinical trials have reported improved gains in clinical

attachment levels with respect to access flap surgery alone [13, 22, 29].

Although EMD has been shown clinically and histologically to induce periodontal tissue regeneration, the mechanism by which it influences cell function still remains unclear. So far, no known growth factors have been detected in EMD. However, amelogenins are known to self-assemble into supramolecular aggregates that form an extracellular matrix with high affinity for hydroxyapatite and collagens, promoting repopulation of periodontal ligament (PDL) fibroblasts during the first weeks after application [8, 10]. Results from a recent study have shown for the first time in humans that EMD is present on denuded root surfaces for up to 4 weeks following periodontal surgery [28].

After precipitation, EMD stimulates the production of transforming growth factor beta-1 and interleukin-6 by PDL fibroblasts [17, 31]. However, the influence of EMD may not be limited to the cementum, since it was reported that EMD also prolongs primary osteoblast growth [14] and stimulates the proliferation of preosteoblasts as well as the differentiation of immature osteoblasts [24]. These observations indicate that EMD may also influence bone formation on titanium implants in the absence of PDL fibroblasts. However, until now no studies were available evaluating the influence of EMD on the proliferation of osteoblasts on titanium implants. Therefore, the present investigation was designed to investigate its possible effects on the attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on titanium implants.

Materials and methods

Titanium implants and cell cultures

A total of 220 sand-blasted and acid-etched (SLA) titanium discs 10 mm in diameter and 2 mm thick (ITI) (Straumann, Waldenburg, Germany) were placed into 24-well plates (Lap Tek Chamber Slide) (Nalge Nunc, Naperville, Ill., USA). Before cell inoculation, McCoy's 5A medium (MCM) (Gibco no. 21017–025) (Life Technologies, Karlsruhe, Germany) supplemented with 25 μg/ml, 50 μg/ml, 100 μg/ml, and 200 μg/ml EMD (Emdogain) (Biora, Malmö, Sweden) was added, and the culture plates were incubated for 30 min. The MCM alone served as control. Human osteoblast-like $SaOs_2$ cells (ATCC no. HTB 85, Manassas, Va., USA) (2×10^4 cells, fourth passage) were suspended in MCM containing 1% penicillin/streptomycin (Gibco, Karlsruhe, Germany) and 10% fetal bovine serum (Gibco) and then inoculated onto the well chambers. Culturing was set at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed after 3 days without the addition of EMD.

Proliferation assay

The proliferation of $SaOs_2$ cells was determined at days 1, 3, and 6 using a CyQuant cell proliferation assay kit (Molecular Probes, Eugene, Ore., USA). The CyQuant GR dye shows strong fluorescence enhancement when bound to cellular nucleic acids. The culture medium was removed at days 1, 3, and 6, and the cells were frozen at −20°C. For the assay, 200 µl of the CyQuant GR solution was added to each well (n=90) and incubated for 5 min at room temperature. The fluorescence of the sample was measured at an emission wavelength of 538 nm and excitation wavelength of 480 nm on a Fluostar-P microplate fluorimeter (SLT, Grödig, Austria).

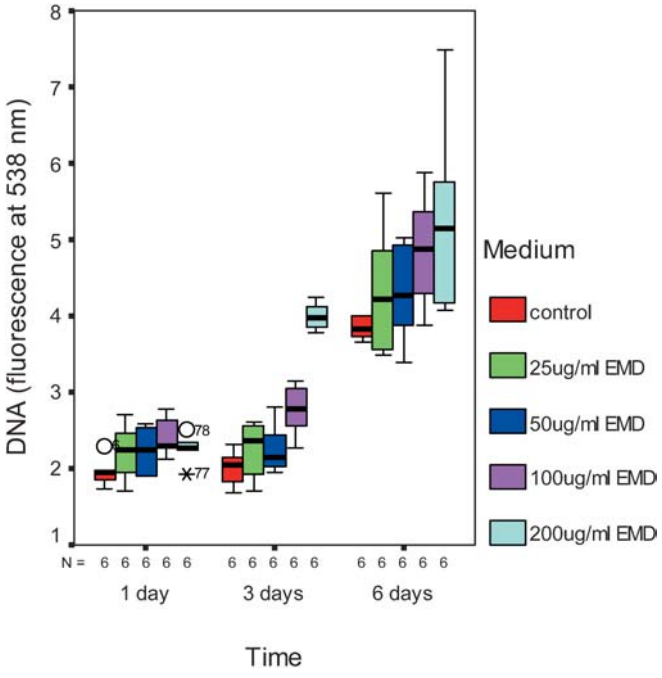

Fig. 1 CyQuant cell proliferation assay: box plots with outliners for the medians and Q1–3 quartiles of DNA content expressed as fluorescence output (emission wavelength 538 nm)

Viability assay

At days 1, 3, and 6, the changes in mitochondrial activity of the $SaOs_2$ osteoblasts was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, Wis., USA). This assay quantifies the ATP, which signals the presence of metabolic active cells and is based on the luciferase-catalyzed reaction of luciferin and ATP. In particular, mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen (Fig. 1). One hundred microliters of CellTiter-Glo reagent were added to the wells (n=90) and incubated for 10 min at room temperature. The luminescent signal was recorded for 1 s per well in a Top Count counter (Canberra-Packard, Dreieich, Germany).

Immunofluorescence staining

Affinity-purified rabbit anti-EMD polyclonal antibodies were kindly supplied by Kamiya Biomedical (Seattle, Wash., USA). These antibodies have been extensively tested in previous experiments [11, 28]. For immunofluorescence staining, titanium discs of all test groups (n=10) were rinsed twice with phosphate-buffered saline (PBS). The discs were incubated with the primary antibody (500 µl per disc, 1:200) for 30 min at room temperature. After three washes, biotinylated secondary goat antirabbit antibody (500 µl per disc, 1:50) was applied for 30 min. This was followed by three further washes with PBS. Fluorescein-conjugated streptavidin was added (500 µl per disc, 1:50). As a negative control, the primary antibody was replaced by nonimmune rabbit serum in the same procedure. Evaluation of the immunofluorescence staining was performed at days 1 and 6 using an Eclipse E 400 fluorescence microscope (Nikon, Düsseldorf, Germany) with an excitation wavelength of 450–490 nm and emission wavelength of 530 nm.

Scanning electron microscopic observation

At days 1, 3, and 6, titanium discs (n=30) were gently washed with PBS to remove cells not attached to the surface and fixed for 30 min with 4% glutaraldehyde in 0.15 M PBS (pH 7.4) at room temperature and then washed in 0.15 M PBS for 15 min. The specimens were dehydrated in increasing concentrations of acetone (from 40% to 100% in 10% steps). After drying in hexamethyldisilazane, the specimens were sputter coated with gold and examined using a model DSM 950 scanning electron microscope (Zeiss, Germany).

Statistical analysis

A version 11.0 software package (SPSS, Chicago, Ill., USA) was used for the statistical analysis. Mean values and standard deviations were calculated for each group. Analysis of variance and post-hoc testing using Bonferroni's correction for multiple comparisons was used within and between groups. Results were considered statistically significant at P<0.05.

Results

During the experimental period, there were no signs of any bacterial or fungal contamination of the well chambers.

Cell proliferation

The DNA content expressed as fluorescence output (emission wavelength 538 nm) is presented in Fig. 1. The most obvious changes in cell proliferation in both test and control groups could be observed between days 3 and 6. In particular, EMD concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml demonstrated similar increases in cell proliferation as in the control group at days 3 and 6, which proved to be statistically significant at both days (P<0.001, respectively). A trend was noted over time that, in contrast to control medium, EMD enhanced cell proliferation in a concentration-dependent manner; however, the differences between test and control groups at different time points were statistically non-significant (P>0.05, respectively).

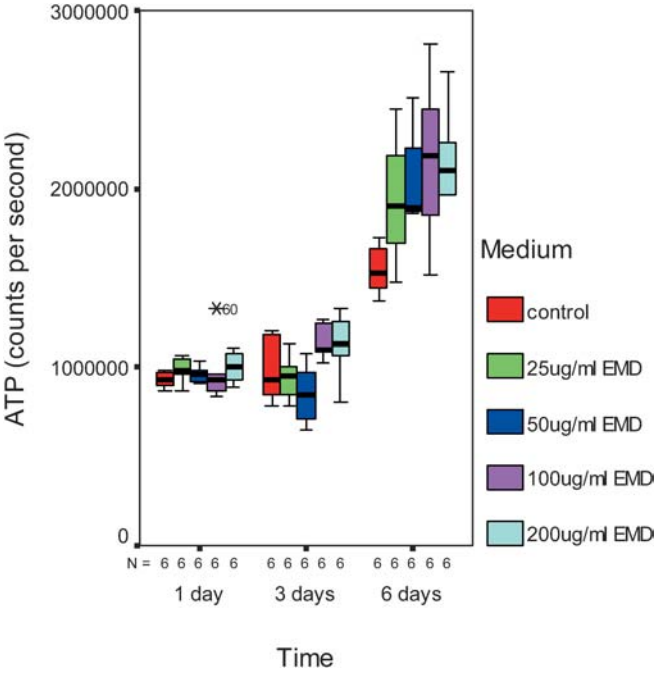

Fig. 2 CellTiter-Glo luminescent cell viability assay: box plots with outliners for the medians and Q1–3 quartiles of mitochondrial activity expressed as luminescent output (counts per s)

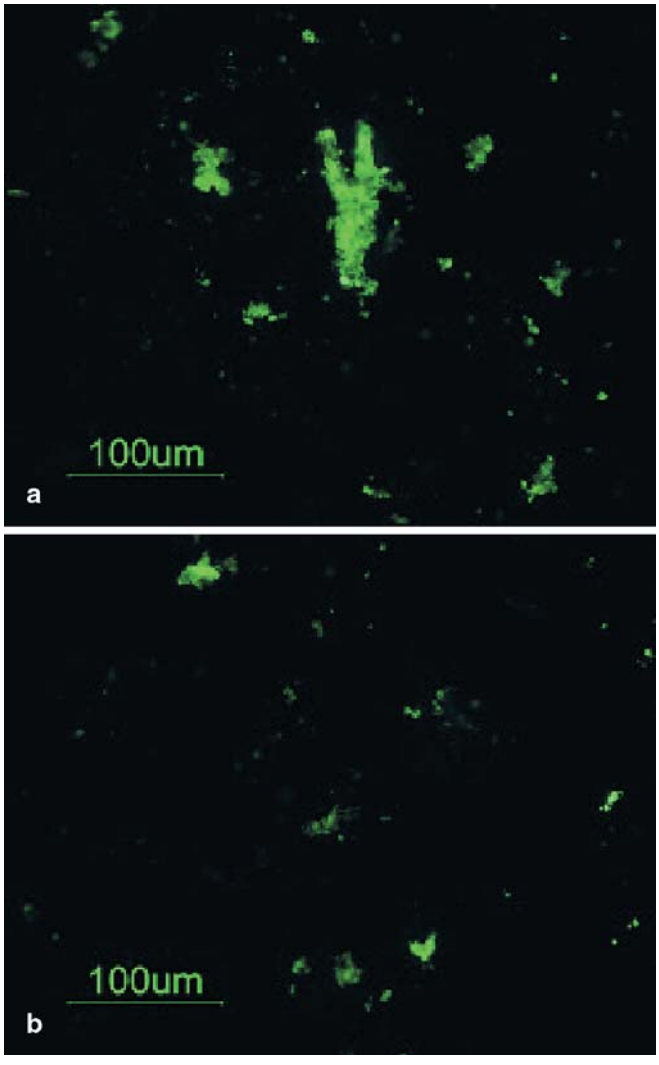

Fig. 3a-b Photomicrographs of amelogenin immunofluorescence staining on an SLA titanium surface. **a** One day after incubation with an $SaOs_2$ osteoblast cell suspension (100 μg/ml EMD). **b** Six days after incubation with an $SaOs_2$ osteoblast cell suspension (100 μg/ml EMD)

Cell viability

The mitochondrial activity of cell cultures expressed as luminescent output (counts per s) is presented in Fig. 2.

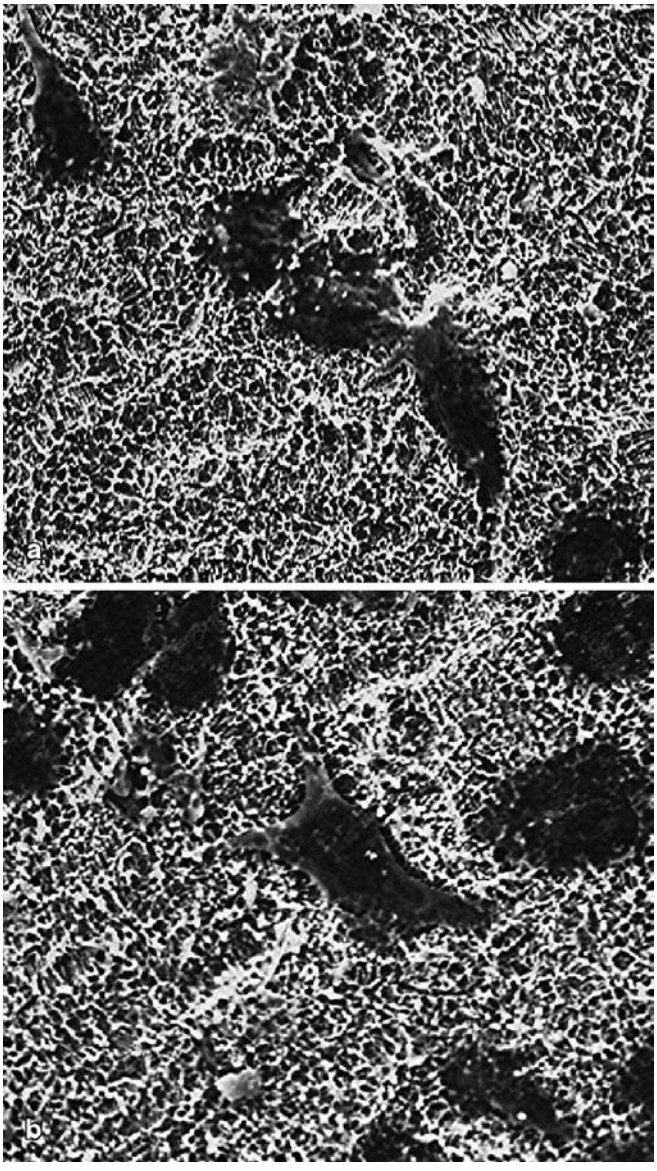

Fig. 4a-b After 1-day incubation, some cells had started to spread, with complete cytoplasmatic extensions of the cell bodies on the titanium surface, shown here by scanning electron microscopy. **a** Control medium (20 kV, original magnification ×500). **b** 100 μg/ml EMD (20 kV, original magnification ×500)

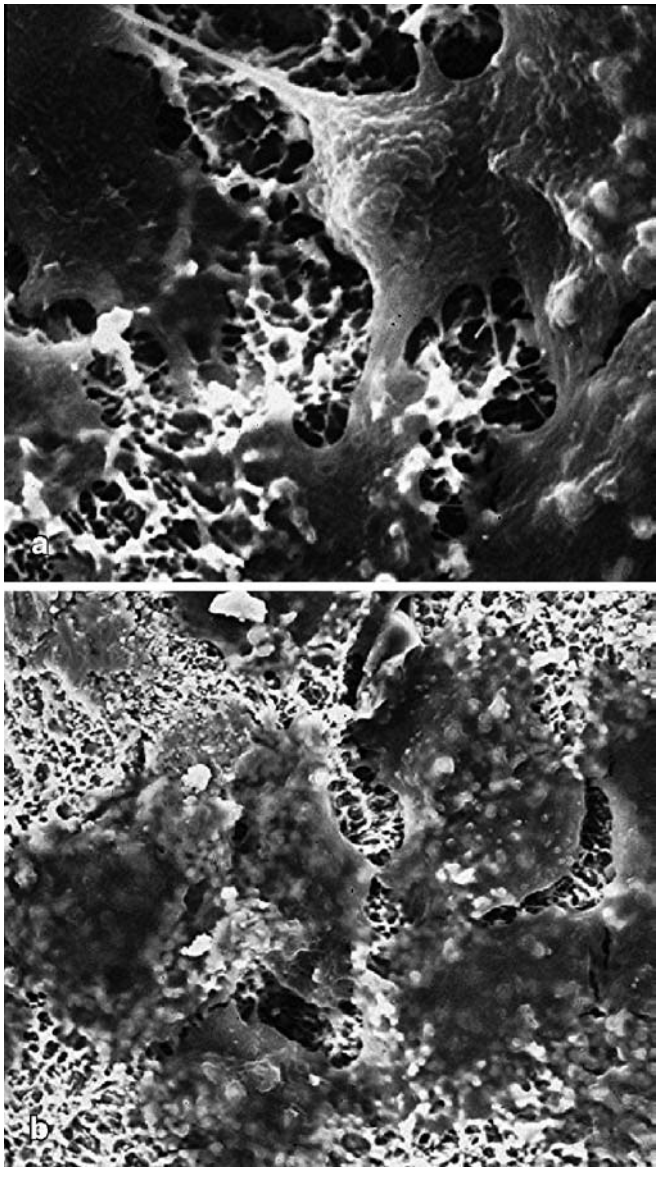

Fig. 5a-b Scanning electron microscopic view of $SaOs_2$ osteoblasts after 3-day incubation. Cell bodies spanned grooves and pits without adaptation to the underlying surface. **a** 25 μg/ml EMD (20 kV, original magnification ×2000). **b** 200 μg/ml EMD (20 kV, original magnification ×1000)

Again, the most obvious changes in cell viability in both test and control groups could be observed between days 3 and 6. In particular, EMD concentrations of 25 μg/ml and 50 μg/ml demonstrated increased cell viability similar to that of the control group at days 3 and 6, which proved to be statistically significant at day 6 ($P<0.001$). The differences between the groups at different time points were statistically nonsignificant ($P>0.05$). However, EMD concentrations of 100 μg/ml and 200 μg/ml resulted in statistically significant increases in cell viability at days 3 and 6 ($P<0.001$, respectively), which were also found to be significantly higher than in the control group at day 6 ($P<0.001$, respectively).

Immunofluorescence staining

Semiquantitative analysis revealed the presence of amelogenin on all titanium discs of the test groups during the entire observation period of 6 days. However, in all test groups, immunofluorescence staining at day 6 was markedly lower than on day 1 (Fig. 3a-b).

Cell morphology

Cell morphology after 1 day showed that $SaOs_2$ osteoblasts appeared to be mostly round in shape. However, some cells had started to spread, with complete cyto-

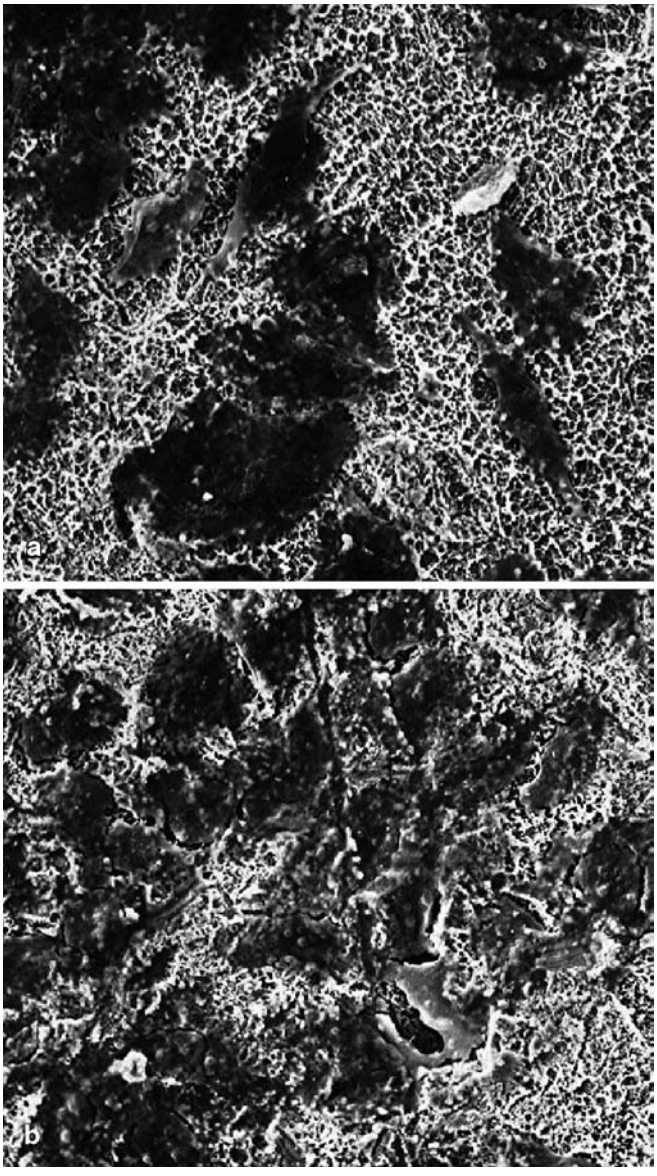

Fig. 6 Scanning electron microscopic view of $SaOs_2$ osteoblasts after 6-day incubation. Cells were mostly flattened, with numerous cytoplasmatic extensions. **a** 50 μg/ml EMD (20 kV, original magnification x 500). **b** 200 μg/ml EMD (20 kV, original magnification ×500)

plasmatic extension of the cell body on the titanium surface (Fig. 4a-b). After 3 days, the cells were mostly flattened, with numerous cytoplasmatic extensions and lamellipodia (Fig. 5a-b). The cell bodies spanned grooves and pits, however, no adaptation to the irregularities of the underlying surface was observed (Fig. 5a-b). After 6 days, cells maintained a star-shaped appearance and approached confluence (Fig. 6a-b). They displayed no orientation. No differences between test and control groups were observed in the morphology of the cells (Fig. 6a-b).

Discussion

The purpose of this study was to investigate the effects of EMD on attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on SLA titanium implants. Although a statistically significant difference from the control medium could merely be observed in mitochondrial activity at day 6 for EMD at 100 µg/ml and 200 µg/ml, a trend was noted over time that EMD enhanced cell proliferation and viability in a concentration-dependent manner. Furthermore, the most obvious changes in both test and control groups could be observed between days 3 and 6.

In the present study, mitochondrial activity was measured using an ATP-based luminescent cell viability assay, which has been reported to be more sensitive than other methods [5, 18, 21]. The luminescent signal generated during cell lysis is proportional to the amount of ATP present. Furthermore, the amount of ATP has been shown to be directly proportional to the number of viable cells present in culture [6].

When interpreting the present results, it was also noted that all investigated EMD concentrations did not seem to have any beneficial effects on cell morphology. In this context, it is important to point out that the cell attachment assay used in our study was similar to techniques described by previous researchers for comparing cellular response to implants with different surface characteristics [2, 4, 5].

Because no previously published data on the effects of EMD on attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on titanium implants are available, it is difficult to compare the present results with other studies. Recent experiments in animals evaluated the effects of EMD on bone healing after guided bone regeneration (GBR) in dehiscence-type osseous defects around dental implants [3]. Surgically treated defects on the buccal aspects of implant osteotomies and titanium implants were randomly treated with EMD, GBR, or EMD+GBR. Untreated defects served as controls. After 3 months, there were no statistically significant differences among the groups in percentage of bone-to-implant contact. However, EMD+GBR combination therapy resulted in a greater area of new bone surrounding the defects than in the control group.

It was hypothesized that the additional use of a barrier membrane may be useful to keep the EMD better in place than with EMD alone. Similar results were reported by Franke Stenport [9], since EMD did not contribute to bone formation around titanium implants. In contrast, it has also been shown that EMD stimulates the proliferation of preosteoblasts as well as the differentiation of immature osteoblasts [24]. However, it is important to point to the results of a recent study which showed that the effects of EMD on osteoblastic cells may depend on cell type.

While EMD did not stimulate mouse ST2 cell growth, it enhanced mouse KUSA/A1 cell proliferation [32]. In this context, it must be emphasized that human osteosarcoma-derived $SaOs_2$ cells have been well characterized

as osteoblast-like cells [20, 23]. However, transformed cell lines have their own limitations, as some of the cell characteristics are different from those of primary cells. Nevertheless, in long-term in vitro mineralization studies, normal human osteoblast cultures responded to implant surfaces in a fashion similar to $SaOs_2$ cells but with approximately two thirds less calcification [1].

The discrepancy noted between these data might be explained by the fact that, even though EMD has a high affinity to hydroxyapatite and exposed collagen fibers on denuded root surfaces [10], it probably does not adsorb to rough titanium surfaces. Indeed, semiquantitative analysis of the present study revealed that immunofluorescence staining of amelogenin on SLA titanium implants at day 6 was markedly lower than on day 1, irrespective of initial EMD concentration. However, it is difficult to estimate whether EMD was reduced during the medium change at day 3 on the one hand or by the metabolic activity of $SaOs_2$ osteoblasts on the other. In this respect, it should also be pointed out that EMD is insoluble in the culture medium, suggesting that it may provide a substratum on the implant surface that is favorable to cell function [11].

Indeed, the results of a recent cell culture study have shown that EMD prolongs osteoblast growth [14]. Primary mouse osteoblasts were plated into 6-well culture plates and incubated in three different groups of media: Dulbecco's modified Eagle medium (DMEM) only as control, DMEM with 25 μg/ml of EMD, and DMEM with 100 μg/ml of EMD. The total cell numbers were calculated at days 3, 7, 10, and 14. It was observed that, at each examination period, the number of cells in the EMD group was significantly higher than in the control group, underlining that EMD may serve as a substratum for cell function. In contrast to the present study, cells were cultured on the culture dish without addition of fetal bovine serum and the medium was not changed.

Commercially available EMD (Emdogain) is dissolved in a propylene glycol alginate (PGA) vehicle in the form of a viscous gel with an acidic pH. At body temperature and neutral pH, the viscosity decreases and the protein precipitates [10]. In this context, it must be emphasized that small shifts in extracellular pH have led to significant changes in the ability of human bone marrow stromal cells to express markers of the osteoblast phenotype in vitro [15]. Thus, the question of the limits of cellular in vitro investigations on acidic substances such as EMD dissolved in PGA has to be raised.

Indeed, the results of a recent study indicated that the viability of human PDL fibroblasts was negatively affected with higher EMD concentrations (75 µg/ml and 100 µg/ml) over time [7]. However, it must be pointed out that results obtained with an in vitro experimental model cannot recreate the complex interactions of cells in vivo. Further studies using controlled experimental in vivo models are needed in order to verify the present results.

Conclusion

Within the limits of the present study, it was concluded that EMD enhanced the cell proliferation and viability of human $SaOs_2$ osteoblasts on SLA titanium implants in a concentration-dependent manner.

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