In Vitro Evaluation of Demineralized Freeze-Dried Bone Allograft in Combination With Enamel Matrix Derivative

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Background: Preclinical and clinical studies suggest that a combination of enamel matrix derivative (EMD) with demineralized freeze-dried bone allograft (DFDBA) may improve periodontal wound healing and regeneration. To date, no single study has characterized the effects of this combination on in vitro cell behavior. The aim of this study is to test the ability of EMD to adsorb to the surface of DFDBA particles and determine the effect of EMD coating on downstream cellular pathways such as adhesion, proliferation, and differentiation of primary human osteoblasts and periodontal ligament (PDL) cells.

Methods: DFDBA particles were precoated with EMD or human blood and analyzed for protein adsorption patterns via scanning electron microscopy. Cell attachment and proliferation were quantified using a commercial assay. Cell differentiation was analyzed using real-time polymerase chain reaction for genes encoding Runx2, alkaline phosphatase, osteocalcin, and collagen 1α1, and mineralization was assessed using alizarinred staining.

Results: Analysis of cell attachment revealed no significant differences among control, blood-coated, and EMD-coated DFDBA particles. EMD significantly increased cell proliferation at 3 and 5 days after seeding for both osteoblasts and PDL cells compared to control and blood-coated samples. Moreover, there were significantly higher messenger ribonucleic acid levels of osteogenic differentiation markers, including collagen 1α1, alkaline phosphatase, and osteocalcin, in osteoblasts and PDL cells cultured on EMD-coated DFDBA particles at 3, 7, and 14 days.

Conclusion: The results suggest that the addition of EMD to DFDBA particles may influence periodontal regeneration by stimulating PDL cell and osteoblast proliferation and differentiation. J Periodontol 2013;84:1646-1654.

KEY WORDS
Adsorption; allograft; blood proteins; bone transplantation; dental enamel proteins; transplantation, homologous.

The goal of regenerative periodontal surgery is to predictably restore the tooth’s supporting apparatus (i.e., root cementum, periodontal ligament [PDL], and bone), which have been lost following periodontal disease or trauma.1-3 Various bone grafts, including autogenous bone, allografts, xenografts, and synthetic alloplasts, have been used with varying degrees of success for the treatment of bone and periodontal defects.4-12 One grafting material that has been particularly effective because of its combination of osteoconduction and osteo-induction is demineralized freeze-dried bone allograft (DFDBA).13-17 The use of DFDBA, alone or in combination with other treatment modalities, has been shown to promote periodontal and bone regeneration and result in substantial clinical improvements evidenced by probing depth reduction, clinical attachment level gain, and defect fill.9,18-26

Similarly, the use of an enamel matrix derivative (EMD) has garnered tremendous notoriety for its ability to regenerate periodontal tissues.27 Although histologic and controlled clinical studies have provided evidence for periodontal regeneration and substantial clinical improvements following the use of EMD in intrabony defects,27 concerns have been expressed regarding the viscous nature

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of EMD, which may not be sufficient to prevent flap collapse in periodontal defects with a non–space maintaining anatomy. Flap collapse may subsequently lead to limitation of the space available for regeneration, thus limiting clinical outcomes. To overcome this potential drawback and improve the clinical results obtained with EMD, recent attempts at combining DFDBA with EMD have been investigated. Whereas a small number of clinical and animal studies reveals a potential benefit for their combination, others have failed to demonstrate an advantage.

Although the limited available data suggest that a combination of EMD and DFDBA may improve regenerative periodontal therapy, no single study has yet evaluated the effect of EMD plus DFDBA on osteoblasts and PDL cell behavior, and thus the effect of cells seeded on EMD-coated DFDBA particles is virtually unknown. Therefore, the aim of the present study is to determine the effect of EMD on osteoblast and PDL cell adhesion, proliferation, and differentiation compared to control and blood-coated DFDBA particles.

MATERIALS AND METHODS

Scanning Electron Microscopy

Scanning electron microscopy was used to visualize the surface topographies of DFDBA particles coated with blood or EMD or left uncoated. DFDBA particles were fixed in 1% glutaraldehyde and 1% formaldehyde. After dehydration with ethanol, samples were critical-point dried, followed by overnight drying. Next, samples were sputter coated with a 10-nm layer of gold and analyzed microscopically using a scanning electron microscope to determine surface variations between samples.

Surface Coating With EMD for In Vitro Experiments

EMD was prepared according to Institute Straumann (Basel, Switzerland) standard operating protocols, as previously described. EMD (30 mg) was dissolved in 3 mL sterile 0.1% acetic acid at 4°C. For experiments, stock EMD was diluted 1:100 in 0.1 M carbonate buffer at 4°C to a working concentration of 100 μg/mL. EMD solution (1 mL) was incubated overnight at 4°C into 24-well culture dishes containing 100 mg DFDBA particles. After incubation, dishes were rinsed twice with 1 mL phosphate-buffered saline (PBS).

Osteoblast and PDL Cell Isolation and Differentiation

Human bone chips were cultured according to an explant model under a protocol approved by the Ethics Committee, Kanton Bern, Switzerland. PDL cells were obtained from the middle third portion of three teeth extracted from three healthy male patients aged 18 to 30 years with no signs of periodontal disease at the Department of Orthodontics, Dental Clinic, University of Bern, as previously described. All patients provided signed informed consent. Primary human osteoblasts and PDL cells were detached from tissue culture plastic using trypsin solution. Cells used for experimental seeding were from passages 4 to 6. Primary osteoblasts and PDL cells were seeded in 24-well culture plates at a density of 10,000 cells (for cell attachment and cell proliferation experiments) and 50,000 cells (for real-time polymerase chain reaction [PCR] and alizarin red experiments). For experiments lasting longer than 5 days, medium was replaced twice weekly.

Adhesion and Proliferation Assays

Primary osteoblasts and PDL cells were quantified using a commercial assay as previously described. Cells were seeded on 100 mg DFDBA per well in 24-well plates at a density of 10,000 cells per well. At 4 and 8 hours for cell adhesion and 1, 2, 3, and 5 days for cell proliferation, cells were washed with PBS incubated with 80 μL aqueous solution dissolved in 400 μL PBS. After 4 hours of culture, cell viability was determined by measuring the absorbance at 490 nm on a 96-well plate reader. Experiments were performed in triplicate with three independent experiments for each condition. Data (± standard error) were normalized to control DFDBA samples.

PCR

Real-time reverse-transcription PCR (RT-PCR) was used to quantify the total ribonucleic acid (RNA) of PDL cell- and osteoblast-related differentiation markers. Total RNA was isolated using a reagent and kit at 3, 7, and 14 days of incubation. Primer and probe sequences for genes encoding alkaline phosphatase ([ALP] Hs01029144_m1), runt-related transcription factor 2 ([Runx2] Hs00231692_m1), collagen 1α1 ([COL1A1] Hs01028970_m1), osteocalcin ([OC] Hs01587814_g1), and GAPDH (Hs03929097_g1) were purchased as predesigned gene expression assays. Real-time RT-PCR was performed using 20 μL final reaction volume of reaction mix as previously described, and 100 ng total RNA was used per sample well. All samples were assayed in triplicate, and three independent
experiments were performed. The count threshold (ΔΔCt) method was used to calculate gene expression levels normalized to GAPDH values and calibrated to control DFDBA particles, except OC expression of PDL cells, which was calibrated to EMD-coated DFDBA particles at 7 days. Data were log-transformed before analysis by two-way analysis of variance (ANOVA) with Bonferroni test, using software.†††

Alizarin Red Quantification
Alizarin red staining was performed to determine the presence of extracellular matrix mineralization after

††† Graphpad, v.4, Graphpad, La Jolla, CA.
21 days. Osteoblasts were seeded at a density of 50,000 cells per 24-well culture dish onto control, blood-coated, or EMD-coated DFDBA particles. After 21 days, cells were fixed in 96% ethanol for 15 minutes and stained with 0.2% alizarin red solution‡‡‡ in water (pH 6.4) at room temperature for 1 hour. Samples representing background staining (DFDBA particles without cells) were treated identically. Alizarin red was dissolved using a solution of 20% methanol and 10% acetic acid in water for 15 minutes. Liquid was then transferred to cuvettes and read on a spectrophotometer§§§ at a wavelength of 405 nm.

Figure 2.
Scanning electron microscopy images demonstrating osteoblast (A, C, and E) and PDL cell (B, D, and F) osteocompatibility with DFDBA particles either coated with blood (C and D) or EMD (E and F) or left uncoated (A and B). No differences in cell morphology could be observed after coating with blood or EMD.

‡‡‡ Sigma-Aldrich, Basel, Switzerland.
§§§ Infinite 200, Tecan Group, Männedorf, Switzerland.
450 nm as previously described. After subtraction of background, absorbance values were normalized to deoxyribonucleic acid content. Data were analyzed for statistical significance using one-way ANOVA with Tukey test.

RESULTS

Scanning Electron Microscopy
To compare DFDBA particles precoated with either blood or EMD, scanning electron microscopy was performed to evaluate the effects of coating on surface characteristics (Fig. 1). All images are shown in duplicate to demonstrate surface variability among DFDBA particles. Many micro- and macrofractures were present in the majority of particles (Fig. 1A). The nano-topography represents a relatively smooth surface with various microporosities present at high resolution (Fig. 1B). Surface coating with blood revealed an abundance of red blood cells (RBCs) and a fibrin matrix on the surface of DFDBA particles (Fig. 1C). The accumulation of hundreds of RBCs could be observed at magnification ×200 (Figs. 1C and 1D). The effect of surface coating with EMD revealed substantial protein deposition on the surface of DFDBA particles (Fig. 1E). Despite widespread protein attachment, variable homogeneity of EMD adsorption was observed (Figs. 1E and 1F). High-resolution images revealed a network of EMD protein fibers spanning over the surface of DFDBA particles, with variable protein density covering the surface (Fig. 1F).

To demonstrate the cell compatibility of DFDBA particles, osteoblasts and PDL cells were observed 24 hours after seeding (Fig. 2). All cells were able to attach and spread on DFDBA surfaces irrespective of surface coating. EMD and blood did not seem to influence cell morphology as assessed by scanning electron microscopy.

Osteoblast and PDL-Cell Attachment and Proliferation
Primary human osteoblasts and PDL cells attached on all control, blood-coated, and EMD-coated DFDBA particles (Figs. 3A and 3B). At all time points, cell number approached 100% cell attachment levels. EMD displayed a significant influence on cell proliferation by significantly increasing the number of osteoblasts (Fig. 3C) and PDL cells (Fig. 3D) at 3 and 5 days after seeding ($P < 0.05$).

Osteoblast Differentiation
Primary human osteoblasts and PDL cells were assessed for ALP, COL1A1, Runx2, and OC gene expression at 3, 7, and 14 days (Fig. 4). ALP levels were significantly higher on EMD-coated DFDBA particles at 3 days for osteoblasts (Fig. 4A) and 14 days for PDL cells (Fig. 4B). COL1A1 messenger RNA (mRNA) levels demonstrated significantly higher expression and at 3 and 7 days after seeding for osteoblasts seeded on EMD-coated DFDBA particles (Fig. 4C) and at all times points for PDL cells seeded on EMD-coated DFDBA particles (Fig. 4D). Analysis of Runx2 gene expression showed significant increases in mRNA levels at both 7 and 14 days after seeding for PDL cells seeded on EMD-coated compared to control and blood-coated DFDBA particles (Fig. 4F). No significant differences

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were observed for Runx2 gene expression in osteoblasts (Fig. 4E). OC mRNA levels showed significant increases for both PDL cells and osteoblasts seeded on EMD-coated DFDBA particles at 7 and 14 days after seeding compared to control and blood-coated particles (Figs. 4G and 4H). To further determine the effects of EMD on osteoblast differentiation, mineralization was assessed via alizarin red staining. At 21 days after seeding, EMD significantly increased alizarin red staining compared to control and blood-coated particles (Fig. 5).

DISCUSSION

EMD is a widely used biologic agent capable of enhancing periodontal wound healing and regeneration. Its in vitro roles have been well documented in both osteoblasts and PDL cells. EMD has a significant influence on cell adhesion, cell proliferation, and cell differentiation of many cell types by mediating cell attachment, spreading, proliferation, and survival, as well as expression of transcription factors, growth factors, cytokines, extracellular matrix constituents, and other molecules involved in the regulation of bone remodeling.

The purpose of this study is to determine the cellular behavior of osteoblast and PDL cells seeded on DFDBA particles in the presence or absence of EMD and blood. For three decades, DFDBA has been used alone or in combination with other regenerative modalities in implant dentistry and periodontal therapy because of its combination of osteoconduction and osteoinduction. The presence of growth factors contained within DFDBA allows for mesenchymal cell migration.
and supports future cell differentiation to bone-forming osteoblasts.

The authors of the current study first sought to characterize the ability for EMD to adsorb to the surface of DFDBA particles. Scanning electron microscopy images demonstrated that EMD was able to adsorb to the surface of DFDBA particles with variable homogeneity (Figs. 1E and 1F). These results are comparable to those described by Gesstrelius et al.,\(^\text{41}\) who demonstrated that EMD forms insoluble spherical complexes on the surfaces of hydroxyapatite, collagen, and denuded root surfaces. The present authors have previously demonstrated that a natural bone mineral (NBM) of bovine origin with a porous structure was able to adsorb EMD to both the surface and innermost portions of NBM particles.\(^\text{33}\) Further research is necessary to evaluate the impact of higher surface macro- and microporosity on protein adsorption of EMD and downstream cellular pathways.

The major component of EMD, amelogenins, are a family of hydrophobic proteins that account for >90% of total protein content and have previously been described as cell-adhesion molecules.\(^\text{41}\) Several reports have confirmed that other molecules within EMD contain integrin-binding regions by demonstrating that PDL cells attach to bone sialoprotein–like molecules through $\alpha_\text{v}\beta_3$ integrins.\(^\text{42}\) In this study, EMD does not influence the ability of either osteoblasts or PDL cells to attach to DFDBA particles. All cells are fully able to attach to DFDBA particles irrespective of surface coating (Figs. 3A and 3B). Furthermore, cell morphology as assessed by scanning electron microscopy did not reveal any discernable differences among treatment groups. Previously, the present authors demonstrated that EMD was able to significantly increase osteoblasts and PDL cells attached on a bovine-derived grafting material;\(^\text{33}\) however, only approximately 50% of cells initially attached to grafting particles. In the present study, the use of DFDBA results in attachment levels of >90% in all treatment modalities, demonstrating the ability for PDL cells and osteoblasts to attach favorably to coated and uncoated DFDBA particles (Figs. 3A and 3B). The most noticeable advantage of precoating EMD onto DFDBA particles is its ability to influence cell differentiation (Fig. 4). In the present study, EMD, when precoated on DFDBA particles, enhances cell differentiation as assessed by real-time PCR and extracellular matrix mineralization by alizarin red staining.

Although previous clinical and animal studies have demonstrated variability among treatments with a combination of DFDBA and EMD, under the present in vitro conditions, EMD is able to stimulate PDL-cell and osteoblast proliferation and differentiation. In a previous in vivo study, it was demonstrated that EMD had no osteoinductive potential by placing capsules containing EMD into calf muscles of mice.\(^\text{31}\) Active DFDBA was shown to be osteoinductive, and its combination with 4 mg EMD resulted in enhanced bone induction. It was concluded that although EMD is not osteoinductive, it is osteopromotive, due in part to its osteoconductive properties.\(^\text{31}\) One explanation for the observed clinical differences may be the variability that exists among DFDBA products caused by processing and sterilization procedures.\(^\text{17}\) It has previously been demonstrated that variability exists among different batches within a tissue bank,\(^\text{17}\) and the effects of these differences on the adsorption of EMD, as well as on the kinetic release of proteins found in EMD, remain unknown. Furthermore, it was also demonstrated that donor age is a likely explanation for the variable osteoinductive potential of DFDBA.\(^\text{43,44}\) Further research is necessary to determine the effects of patient age as well as processing and sterilization procedures of DFDBA as possible reasons for variability in EMD adsorption.

**CONCLUSIONS**

Results from the present study demonstrate that DFDBA particles precoated with EMD enhance PDL cell and osteoblast proliferation and differentiation. Although the outcome of precoating EMD onto bone grafts remains unknown, the results from the present study suggest that the combination of EMD with DFDBA could prove to be an important tool for enhancing periodontal regeneration.
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