Clin Oral Invest (2012) 16:745–754
DOI 10.1007/s00784-011-0570-7

ORIGINAL ARTICLE

Human periodontal ligament fibroblasts stimulated by nanocrystalline hydroxyapatite paste or enamel matrix derivative. An in vitro assessment of PDL attachment, migration, and proliferation

Adrian Kasaj · Brita Willershausen · Rüdiger Junker · Stefan-Ioan Stratul · Mirko Schmidt

Received: 21 April 2010 / Accepted: 17 May 2011 / Published online: 7 June 2011

Abstract We determined the effects of soluble or coated nanocrystalline hydroxyapatite paste (nano-HA) and enamel matrix derivative (EMD) on proliferation, adhesion, and migration of periodontal ligament fibroblasts (PDLs). Cultured PDLs were stimulated with nano-HA paste or EMD in a soluble form or were coated to the surface of cell culture dishes. Proliferation of PDLs on coated nano-HA and EMD was quantified by various methods including bromodeoxyuridine (BrdU) incorporation and Western blot. Cell migration was investigated in a modified Boyden chamber. The surface integrin profile of PDLs was determined using an integrin-specific ELISA, and integrin-specific signaling was measured by immunoblotting of phosphorylated focal adhesion kinase (FAK). Coated nano-HA stimulated PDL proliferation to a larger extent as compared with coated EMD. PDL migration towards a nano-HA or EMD gradient was more efficiently mediated by soluble EMD as compared with nano-HA but vice versa, adhesion of PDLs to compound-coated dishes was more effectively mediated by nano-HA as compared with EMD. Mechanistically, majorly integrin $\alpha 5\beta 1$-mediated adhesion of PDL and both coated compounds mediated a significant increase in FAK activation though to a different extent. Current findings offer two different modes of action for EMD and nano-HA paste. EMD efficiently acts as a chemoattractant in its soluble form, while nano-HA paste effectively serves as a synthetic extracellular matrix component in its coated form. Our findings suggest that EMD and nano-HA paste display different molecular characteristics and apply alternative routes to mediate their beneficial effects on periodontal tissues.

Keywords Bone grafts · Enamel matrix derivative · Nanocrystalline hydroxyapatite · Periodontal ligament fibroblasts (PDLs) · Periodontal regeneration

A. Kasaj (✉) · B. Willershausen
Department of Operative Dentistry and Periodontology,
Johannes Gutenberg-University,
Augustusplatz 2,
55131 Mainz, Germany
e-mail: Kasaj@gmx.de

R. Junker
Department of Implantology and Periodontology,
Radoud University Nijmegen Medical Centre,
Nijmegen, The Netherlands

S.-I. Stratul
Department of Periodontology,
Victor Babes University of Medicine and Pharmacology,
Timisoara, Romania

M. Schmidt
Institute of Neurology (Edinger Institute),
Johann Wolfgang Goethe-University, School of Medicine,
Frankfurt, Germany

Introduction

The aim of periodontal therapy is to control periodontal tissue inflammation and to produce predictable regeneration of periodontium lost as a result of periodontal disease. A variety of treatment approaches for the restoration of lost periodontal structures have been suggested by several authors [1–5]. Among these different treatment modalities, different types of bone replacement grafts, guided tissue regeneration (GTR), enamel matrix derivative (EMD), growth factors, or combined approaches were employed with varying success for the regeneration of tooth-

supporting structures. Although GTR is one of the best documented regenerative approaches, the clinical application is often difficult, and the outcome of regenerative therapy appears to be affected by several confounding factors [6]. Thus, the bacterial contamination of exposed barrier membranes and the underlying healing tissues have been associated with reduced clinical outcomes [7]. An alternative approach to obtain periodontal regeneration is the use of bone replacement graft techniques. These techniques are based on the concept of filling the intrabony defect with a number of grafting materials to stimulate bone repair, including autografts, allografts, xenografts, and alloplasts. The use of different types of alloplastic grafts for regenerative periodontal treatment has been shown to significantly improve probing depths and clinical attachment levels [8, 9]. However, histological analysis revealed no or only an unpredictable amount of periodontal regeneration following the application of alloplastic materials [10, 11].

Recently, a fully synthetic nanocrystalline hydroxyapatite (nano-HA) paste (Ostim®, Heraeus Kulzer, Hanau, Germany) containing 65% water and 35% of nanostructured apatite particles has been introduced for augmentation procedures in osseous defects [12, 13]. Advantages of such a nanostructured material in comparison to bulk material are the close contact with surrounding tissues, quick resorption characteristics, and a high number of molecules on the surface. The nano-HA paste has already been used for the treatment of various types of metaphyseal fractures such as the calcaneus and tibia in orthopedic surgery [14], as well as for tooth perforations [15], jaw cysts [16], and peri-implantitis lesions [17]. Findings from recent clinical studies have indicated that the treatment of periodontal intrabony defects with nano-HA paste may provide additional clinical benefits in terms of clinical attachment level gains and probing depth reductions as compared with open flap debridement alone [18, 19]. Experimental studies demonstrated that nanosized ceramics may represent a promising class of bone graft substitutes due to their improved osseointegrative properties [20, 21]. In particular, experimental animal studies demonstrated an undisturbed osseous-integration and complete resorption of the material within 12 weeks [21]. Recent research showed that synthetic nanostructured HA enhanced osteoblast functions [22] and increased biocompatibility for microvascular endothelium [23]. However, there is still only limited data on the molecular mechanisms mediated by nano-HA paste, particularly its effects on cultured cells derived from adult human periodontal tissues [24, 25]. Current evidence suggests that periodontal ligament (PDL) fibroblasts play a major role in the periodontal wound healing process [26]. Since the attachment of PDLs to bone graft materials (BGM) is of importance to periodontal therapy involving placement of BGM, we investigated the interactions of cultured human PDL fibroblasts with a novel nanostructured bone replacement graft. Comparison of these results with those of EMD, a material already well-analyzed in this context, allowed us to determine possible similarities or differences in the mechanisms mediated by both materials. The current study was designed to determine how nano-HA paste affects the attachment, migration, and proliferation of PDL fibroblasts and further, to suggest a molecular explanation for these phenotypes.

Materials and methods

Reagents

Nano-HA paste (Ostim®) was obtained from Heraeus Kulzer (Germany)). The paste consists of a suspension of pure HA in water prepared by a wet chemical reaction. After completion of the paste, the HA content yields 35%. The average size of the needle-shaped HA crystals are about 18 nm; the atomic ratio of calcium-phosphorus is 1.67. The material is characterized by a large bioactive specific surface area of 106 m^2g^{-1} [27]. For current experiments, hydroxyapatite paste was dissolved in phosphate buffered saline (PBS, pH 5.2) to a final concentration of 10 mg/ml used in cell adhesion experiments. Other materials were dissolved in standard cell culture PBS. Nano-HA paste was extensively rinsed with standard PBS after coating but prior to use for adhesion or cell proliferation experiments. In migration assays, nano-HA solution was diluted to a final concentration of 100 μg/ml. The Emdogain® (EMD) was supplied by Straumann (Switzerland). EMD is a product purified from porcine enamel matrix protein extracts containing 90% amelogenins. The remaining 10% include proline-rich non-amelogenins, tuftelin, tuft protein, and serum proteins [28]. Since EMD is highly hydrophobic, it is suspended in a vehicle of propylene glycol alginate. To create a viscous solution required for our experiments, lyophilized EMD was mixed with 3 ml of a 7.5% acetic solution to achieve a stock solution of 10 mg/ml that was used for coating of cell culture dishes in migration studies as well as cell adhesion and proliferation assays.

Cell culture and media

Human PDL fibroblasts were obtained from healthy human periodontal tissues isolated from third molars extracted for orthodontic reasons from three young volunteers (two males and one female aged 14 to 18 years). Prior to extraction, patients were informed about the study and agreed to experimental use of the extracted teeth. The study was conducted in accordance with the second Helsinki

Declaration. In brief, PDL tissue fragments were mechanically removed under sterile conditions by scraping the middle third of the root surface with a sharp blade. Tissue explants were maintained in Dulbecco`s modified Eagle`s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), 1% fungizone (Sigma, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; PAA, Pasching, Austria). Within 3 weeks, the PDL explants were successfully forming primary cultures with a sufficient number of new cells. Cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% air. Tissue culture medium was changed every 2 days until confluence (approximately 7×10^4 cells/cm^2) was reached, and cells were passaged at a 1:2 split ratio following trypsinization with 0.05% trypsin (Invitrogen, Carlsbad, CA, USA). Cell cultures were tested on a regular base to be free of mycoplasma, and cell growth was monitored by phase-contrast microscopy. All cells demonstrated a fibroblast-like morphology and a comparable rate of proliferation. In order to investigate whether the cells were not merely gingival fibroblasts, cells were tested for alkaline phosphatase (ALP). As most of the cells were strongly positive for ALP, it was assumed that the cells were indeed periodontal fibroblasts. PDL fibroblasts were used for experiments between passages 4 and 9, and all experiments were performed in triplicate.

Cell attachment assay

In order to evaluate the potential of nano-HA paste and EMD to serve as synthetic extracellular matrices (ECM), cell attachment assays using PDLs were performed. Coated dishes were prepared by covering suspension dishes (Greiner, Frickenhausen, Germany) with 10 mg/ml EMD, nano-HA paste, or fibronectin (Upstate, Charlottesville, VA, USA) in PBS at 4°C overnight. The ECM protein fibronectin served as a positive control as this protein is a strong mediator of cellular adhesion [29]. Prior to use, plates were washed extensively with PBS, blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 4°C and were washed again with PBS. PDLs (3×10^5) were seeded on coated or uncoated dishes and incubated at 37°C for 1 h. Unbound cells were removed by washing with PBS for three times. Attached cells were stained with 0.2% crystal violet (Sigma, St. Louis, MO, USA) in 10% ethanol, lysed, and quantified by determination of absorbance in a microplate reader (Tecan M200, Munich, Germany) at 565 nm. The assays were performed in triplicate and presented as the average of three data points as plotted in arbitrary units.

BrdU proliferation assay

The PDL proliferation rate was determined by BrdU incorporation over night using a cell proliferation ELISA (Roche, Basel, Switzerland) according to the manufacturer's guidelines. In brief, PDL fibroblasts were plated on 35-mm Petri dishes coated with various compounds (BSA, nano-HA paste, EMD, or fibronectin) at 5×10^3 cells per cell culture dish in DMEM with 10% FBS. The culture plates were incubated for 24 h with BrdU at 37°C in a 5% CO_2 atmosphere. Next day, cells were harvested and BrdU incorporation measured using the BrdU ELISA. Each experiment was performed in triplicate for each experimental group (BSA, nano-HA paste, EMD, and fibronectin).

MTS assay for metabolic activity

The amount of PDLs was determined using the CellTiter 96 Aqueous One solution assay (Promega, Mannheim, Germany) which is based on the conversion of the MTS tetrazolium compound into a colored formazan product. PDL fibroblasts were plated in DMEM with 10% FBS onto 96-well plates coated with various compounds (BSA, EMD, nano-HA paste, or fibronectin) at 5×10^3 cells per well and allowed to attach over night. Subsequently, medium was exchanged, and the MTS compound was added into each well for 3 h at 37°C after which the absorbance was measured at 490 nm. Each experiment was performed in triplicate for each experimental group (BSA, EMD, nano-HA paste, and fibronectin) and data plotted as mean±SD.

Cell migration assay

PDL migration was measured in a modified Boyden chamber by using a FluoroBlokTM 24-multiwell insert system (BD, Heidelberg, Germany). The inserts of the chamber consist of a light-opaque polyethylene membrane with 8.0-μm-sized pores blocking the transmission of light within the range of 490–700 nm. Some 1×10^5 cells per chamber were loaded in 500 μL DMEM (Invitrogen, Carlsbad, CA, USA). The outer well was loaded with 750 μL DMEM containing 100 μg/ml EMD, nano-HA paste, BSA (Sigma, St. Louis, MO, USA), or alternatively, 5 ng/ml bibasic platelet-derived growth factor (PDGF-BB, Peprotech, Hamburg, Germany). Cells were allowed to migrate for 24 h under standard conditions at 37°C. Subsequently, cells were fixed in 4% paraformaldehyde (PFA) in PBS and stained with 0.5 μg/ml 4`, 6-damino-2-phenylindole dihydrochloride (DAPI) (Sigma, St. Louis, MO, USA). The number of cells migrated through the FluoroBlok inserts were counted under a fluorescence microscope (Zeiss, Jena, Germany) and plotted as an average of three test samples.

Determination of cell surface integrins

Expression of surface integrins in PDLs was determined using the integrin-mediated cell adhesion combo (Chem-

icon, Temecula, CA, USA) according to the manufacturer`s guidelines. In brief, 1×10^5 cells were seeded per well coated with various integrin antibodies as supplied in the kit. After 1 h incubation at 37°C, unbound cells were removed by washing with PBS, and adherent cells were stained with 0.2% crystal violet (Sigma, St. Louis, MO, USA) in 10% ethanol, lysed, and quantified by determination of absorbance in a microplate reader at 565 nm (Tecan M200, Munich, Germany). Results are shown in arbitrary units.

Immunoblotting

For detection of proteins in Western blots, monoclonal mouse anti-phospho-Erk (E-4) and anti-actin (C-2) as well as polyclonal rabbit anti-Erk2 (C-14), anti-pY-FAK (Tyr-397), and anti-FAK (A-17) were obtained from Santa Cruz Biotechnology. Further reagents used were rabbit monoclonal anti-Akt and anti-pS-Akt (Ser473) antibodies (Cell Signalling Technology).

Cells were incubated in the presence of standard culture medium, PBS, nano-HA paste, or EMD in standard culture medium. Protein samples were analyzed by SDS-PAGE using an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) in combination with precast NuPAGE 4–12% or 10% Bis–Tris gels (1 mm; NuPAGE 4–12% or 10% Bis–Tris gels, Invitrogen) at 200 V according to the manufacturer's guidelines. Following electrophoresis, proteins were blotted to a PVDF membrane and were incubated for at least 1 h in blocking buffer (5% BSA and 1% Tween-20 in Tris-buffered saline. Membranes were incubated overnight with appropriate dilutions of primary antibody in blocking buffer. The next day, membranes were washed and incubated for 1 h with alkaline-phosphatase-conjugated secondary antibody solution in blocking buffer (dilutions, anti-mouse antibody 1:3,000 and anti-rabbit antibody 1:5,000; Sigma, St. Louis, MO, USA). After additional washing steps, antibody complexes were visualized on film by Immun-Star AP substrate (Bio-Rad, Germany).

Data analysis

Statistical analysis was performed with Student's t test. Differences were considered significant at a p value<0.05 (two-tailed).

Results

Effects of nano-HA paste and EMD on PDL adhesion

PDL fibroblasts were seeded on plastic dishes coated with nano-HA, EMD, and fibronectin (as a positive control), and the adhesion potential of these compounds was assessed (Fig. 1). Fibronectin was most effective in this setting but also nano-HA-mediated adhesion (about half the value of the positive control fibronectin). The strength of adhesion of PDL fibroblasts to EMD was low but still doubled the amount reached by the negative control BSA. Taken together, cellular adhesion of PDLs towards nano-HA paste-coated cell culture dishes was stronger as compared with EMD. However, both materials did not reach the adhesive potential of the extracellular matrix component fibronectin.

Nano-HA paste and EMD stimulate PDL proliferation

In order to determine whether coated nano-HA or EMD affect cell proliferation, PDLs were seeded on compound-coated plastic dishes, and cell proliferation was assessed by incubation with BrdU that incorporated into the DNA of

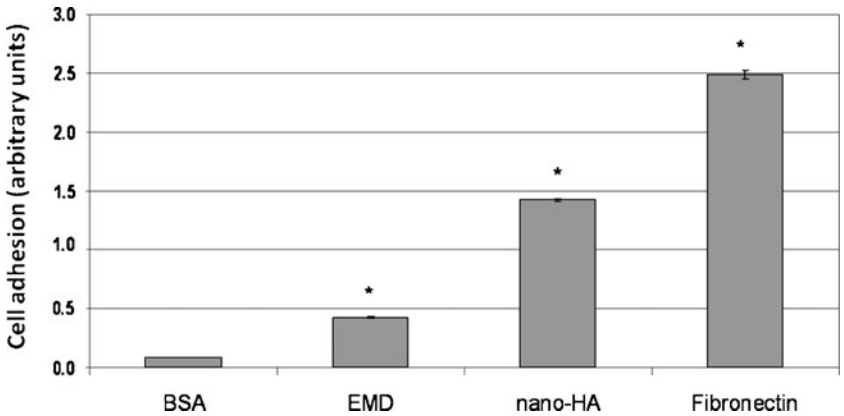

Fig. 1 Effect of nano-HA, EMD, and fibronectin on PDL adhesion. Cells were cultured on 35-mm cell culture dishes coated with the various compounds at 5×10^3 cells per dish in DMEM with 10% FBS. Cell adhesion was analyzed by crystal violet staining and subsequent colorimetric quantification. *Error bars* represent the standard deviation of the mean (n=3) as plotted in arbitrary units. *Significantly different from negative control (p<0.05)

proliferating cells. Cell proliferation was induced in a substrate-dependent manner (Fig. 2), and the maximal effect was observed once cells were seeded on nano-HA paste. Cells grown on coated EMD proliferated slower, roughly comparable to the control fibronectin. The negative control BSA did not yield a significant effect on proliferation.

To further investigate the effect of nano-HA and EMD on PDL proliferation and as an independent proliferation control, we used the MTS assay to measure cell viability. Data suggest that nano-HA and EMD significantly increased the metabolic rates of PDL fibroblasts if compared with the negative control BSA (Fig. 3). As detected above, adhesion of PDL to nano-HA induced proliferation most efficiently, whereas EMD and the ECM protein fibronectin were comparable but stimulated proliferation to a smaller extent than nano-HA. Thus, nano-HA and EMD demonstrated a beneficial effect on PDL viability and proliferation.

Pro-proliferative cues in several cell types are often transduced by molecular signaling via ERK1/2 and Akt kinases; therefore, we hypothesized that cultivation of PDLs on nano-HA or EMD may engage ERK1/2 and Akt activation. Phosphorylation, as a measure of ERK1/2 activation in PDLs, was detected by Western blot analysis using specific antibodies against phosphorylated ERK1/2. The amount of phosphorylated ERK1/2 increased upon cultivation of PDLs on nano-HA, EMD, and fibronectin (Fig. 4). The phosphorylation level of ERK1/2 in PDLs seeded on nano-HA was higher as compared with EMD and fibronectin, reflecting data obtained by BrdU and MTS proliferation assays. Likewise, activation of Akt in PDLs was detected by Western blot using antibodies recognizing phosphorylation of Akt at serine 473. Akt is linked to PI3K signaling, another major pathway of intracellular signal transduction that has a major role in the transmission of proliferation in a variety of cells [30]. Results demonstrated that nano-HA and EMD as well as fibronectin as a positive control stimulated Akt phosphorylation to a significant extend. The phosphorylation of Akt in PDLs upon seeding on nano-HA-coated dishes was higher as compared with EMD and fibronectin, comparable to data obtained above (Fig. 4).

Influence of nano-HA paste and EMD on PDL migration

The influence of nano-HA and EMD on PDL adhesion and proliferation raised the question if these proteins might affect cell migration as well. This prompted us to analyze whether or not both materials act as chemoattractants if applied in a soluble form; therefore, we loaded the lower chamber of a modified Boyden chamber with cell medium supplemented with the soluble forms of both compounds or 5 ng/ml PDGF-BB, a potent inductor of fibroblast migration. PDLs were seeded into FluoroBlok chambers and were allowed to migrate for 24 h. Subsequent analysis revealed that the negative control BSA attracted less than 20 cells into the filter, whereas EMD with more than 60 counts promoted cellular migration significantly stronger than BSA. PDGF-BB as the positive control was most effective in this setting and attracted about 125 cells per microscopic field. Soluble nano-HA paste on the other hand did not induce significant cell migration, barely above the negative control BSA. Taken together, our findings suggest that EMD may act as a chemoattractant but nano-HA paste does not (Fig. 5).

Molecular mechanism underlying nano-HA paste and EMD effects in PDLs

The effects of nano-HA and EMD on PDL adhesion, PDL migration, and PDL proliferation raised the question on the

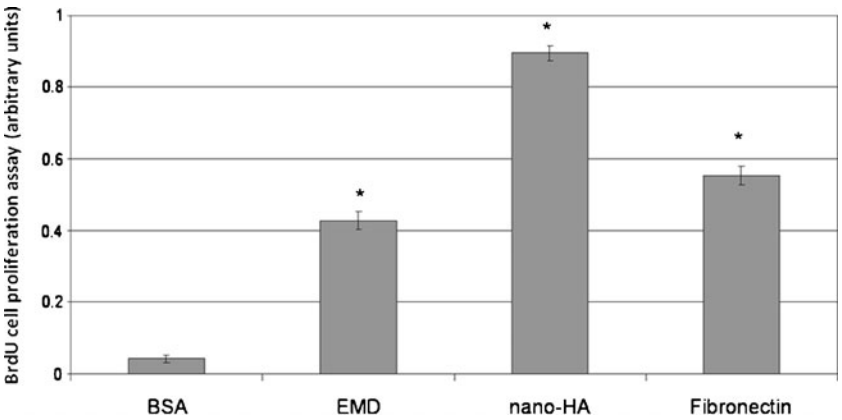

Fig. 2 Effect of nano-HA, EMD, and fibronectin on PDL proliferation. Cells were cultured on 35-mm cell culture dishes coated with the various compounds at 5×10^3 cells per dish in DMEM with 10% FBS. Proliferation was determined by BrdU incorporation. *Error bars* represent the standard deviation of the mean (n=3). *Significantly different from negative control (p<0.05)

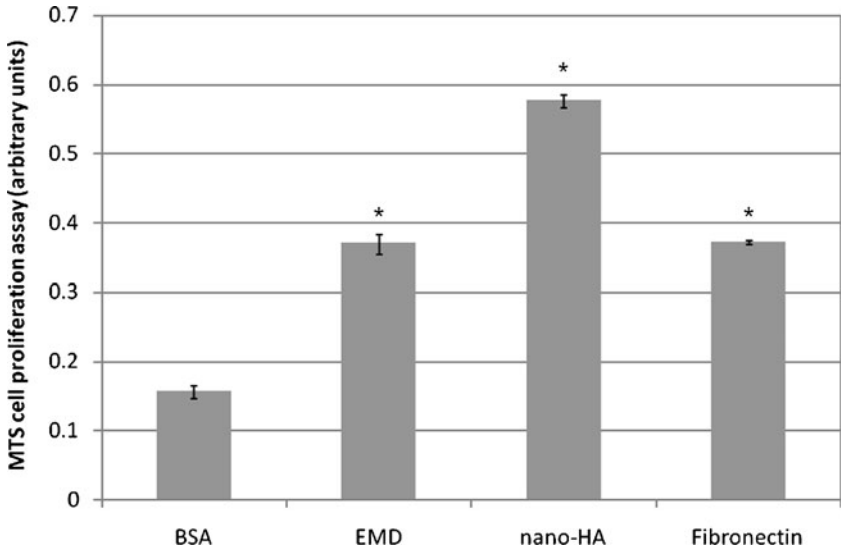

Fig. 3 Effect of nano-HA, EMD, and fibronectin on PDL viability. Cells were cultured on 35-mm cell culture dishes coated with the various compounds at 5×10^3 cells per dish in DMEM with 10% FBS. PDL fibroblasts viability that is directly linked to proliferation was determined by an MTS assay. Data are shown as the mean±SD. *Significantly different from negative control ($p<0.05$)

molecular basis of these observations. Typically, transmembrane receptors of the integrin type mediate cell adhesion; therefore, we analyzed the surface expression profile of integrin receptors on PDLs by ELISA. Our results revealed that PDLs express a wide range of integrins on their surface (Fig. 6); however, $\alpha_5\beta_1$ and both of its subunits, α_5 and β_1, displayed a higher amount of surface expression as compared with other integrins. This indicates that $\alpha_5\beta_1$ might be a major mediator of PDL adhesion. In order to analyze if the adhesive effects of nano-HA and EMD are mediated by receptors of the integrin type, lysates of PDLs seeded on compound-coated dishes were analyzed by Western blot for the phosphorylation level of focal adhesion kinase (FAK), a major component of integrin signaling. As a measure for integrin-specific activation of this kinase we analyzed phosphorylation of tyrosine residue 397, the autoactivation site of FAK (FAKpY397). Clearly, nano-HA paste and EMD both induced phosphorylation of FAK at this site (Fig. 7a), with nano-HA being more effective in this setting (Fig. 7b), indicating that both compounds affect PDLs in an integrin-dependent manner.

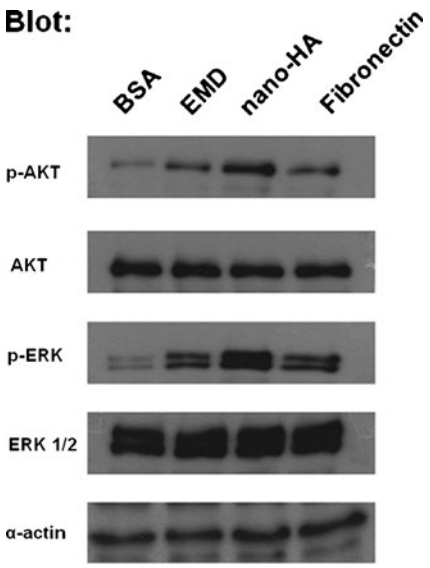

Fig. 4 Effect of nano-HA, EMD, and fibronectin on PDL proliferation. Cells were cultured on 35-mm cell culture dishes coated with the various compounds at 5×10^3 cells per dish in DMEM with 10% FBS. Proliferation was determined by Western blot-based detection of phospho-ERK1/2 and phospho-Akt (Ser473) antibodies as a direct measure for the activation of the proliferation-linked kinases ERK1/2 and Akt. α-Actin served as an internal control to ensure equal loading of protein in each lane

Discussion

A variety of materials are used in the treatment of periodontal disease to promote periodontal wound regeneration and repopulate the damaged area by desirable cells. Recent studies identified nanostructured HA paste as a promising class of bone graft substitutes [13, 31]. However, the mechanism how this material promotes periodontal regeneration remained elusive. The objective of this study was to determine how nanostructured HA paste affects PDL adhesion, migration, and proliferation; therefore, we used the compounds in this study in a soluble or a surface-coated form. The preparation and concentration of EMD was described in previous in vitro studies [32, 33]. Our results indicate that nano-HA paste is a strong stimulator of PDL attachment. Furthermore, data suggest that nano-HA paste acts as a stimulator of cell migration and proliferation. Adhesion assays revealed a stronger increase in cell attachment in case of nano-HA paste if compared with EMD. An explanation might be provided by the composition of this specific material. Although the active ingredient of EMD, amelogenin, was described to mediate cell

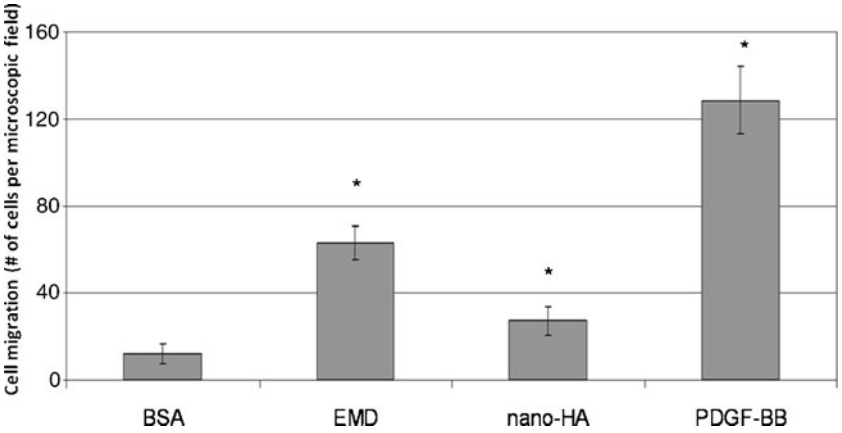

Fig. 5 Effect of nano-HA, EMD, and PDGF-BB on PDL fibroblast migration. The cells were plated at 1×10^5 cells per well on the top of the inlay of a Boyden chamber and were allowed to migrate for 24 h towards a gradient of soluble nano-HA, EMD, or PDGF-BB. *Error bars* represent the standard deviation of the mean ($n=3$). *Significantly different from negative control ($p<0.05$)

adhesion [34], nano-HA paste is much more effective in this context. This might be explained by the fine size of the nanocrystalline HA particles, which exhibit high solubility, high surface reactivity, high surface energy, and high capacity for ion exchange reaction [35]. Our findings are supported by Webster et al. [20], who reported an increased initial attachment of osteoblasts grown on nanophase HA compared with conventional ceramics. Similarly, Mateus et al. [36] showed that nanohydroxyapatite microspheres have the ability to promote adhesion and proliferation of osteoblasts. Furthermore, it was shown that proteins interact differently with nanophase materials compared with conventional ceramics of similar chemistries [37].

The response of examined PDL fibroblasts towards surface-coated EMD showed an increase in proliferation as documented before [32, 38]. Interestingly, the proliferation of PDL on coated nano-HA paste was duplicated as compared with the tested ECM protein or EMD. In contrast to this observation, our previous study [39] demonstrated a twofold less proliferation potential of nano-HA paste in comparison to EMD. However, in this previous work, PDLs were stimulated with nano-HA and EMD in a soluble form, a condition where nano-HA affects PDLs much less efficient as compared with EMD (Fig. 5), whereas proliferation in the present study has been assessed using nano-HA and EMD in a surface-coated form. One reason for the opposing findings obtained by using nano-HA in a soluble or a coated form might be the enhanced adhesion of PDLs on coated nano-HA as described in our present study. This results in an increase in integrin signaling as measured by increased autophosphorylation of FAK in immunoblots. Previous in vitro studies demonstrated the ability of integrins to mediate cell attachment to extracellular matrices [40, 41]. Alterations in cellular adhesion result in an increase in cell proliferation [42]. Signaling pathways downstream of integrins are cross-connected to those of certain receptor tyrosine kinases that induce cell proliferation. Thus, the enhanced effects of nano-HA and EMD

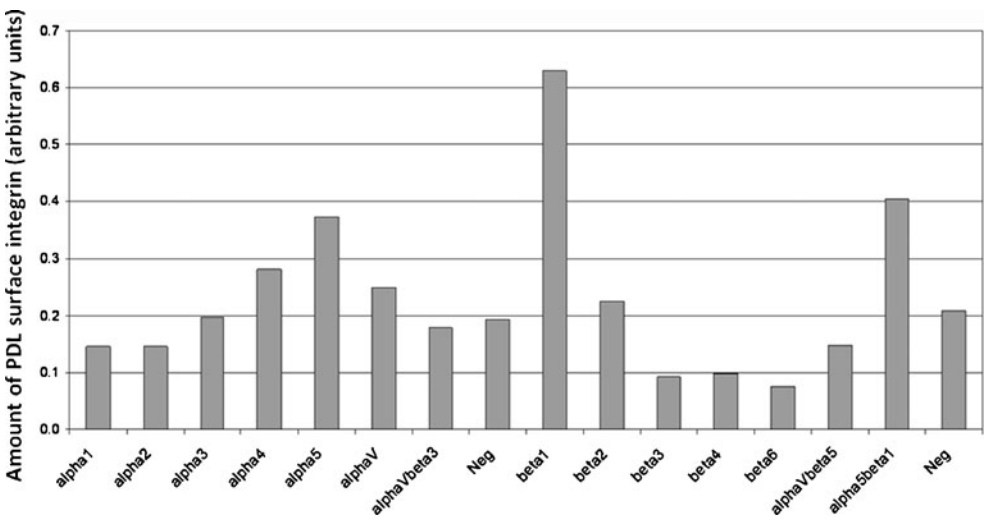

Fig. 6 Expression of the surface expression profile of various integrins on PDL fibroblasts by ELISA. The results are expressed in arbitrary units as compared with negative control

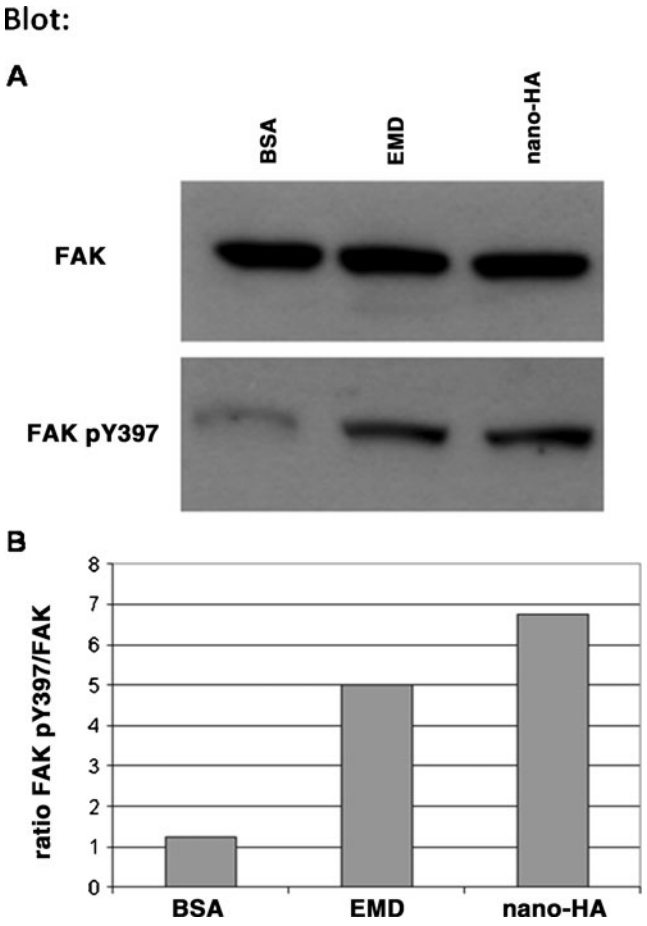

Fig. 7 Phosphorylation of focal adhesion kinase (FAK) in response to stimulation by nano-HA and EMD. Cells were cultured on 35-mm cell culture dishes coated with the various compounds at 5×10^3 cells per dish in DMEM with 10% FBS. **a** The amounts of FAK and its autophosphorylated form FAKpY397 in human PDLs after stimulation with BSA, nano-HA, or EMD. **b** Densitometric analysis of the immunoblots indicating the ratio of phosphorylated FAKpY397 as compared with the total FAK level, which is a measure for integrin-specific activation of FAK

might be linked via integrin-receptor tyrosine kinase cross-talk. Indeed, there is evidence that integrins transduce signaling pathways by phosphorylation of focal adhesion kinases that leads to the activation of ERK1/2 [43]. This implicates that surfaces mediating strong cellular adhesion might increase cell proliferation as well. Consequently, we observed that nano-HA increases cellular proliferation much stronger as compared with EMD, which reflects the cell adhesion capacity of both compounds. The mitogenic effect of nano-HA on cell proliferation was mediated by activation of ERK1/2 and Akt, which was more pronounced than that following exposure to EMD. These findings are supported by our previous research demonstrating that increased proliferation rates of PDLs in the presence of nano-HA paste are linked to activation of ERK1/2 and Akt [39, 44]. An explanation for this phenomenon might be the cellular response to the microstructure of nano-HA paste. As described by Garcia et al. [45], there is an integrin-mediated cross-connection between the cellular surface and the cellular activity. The surface composition of the nano-particles containing nano-HA paste might be the adequate stimulus for integrin-mediated cell proliferation. Indeed, de Ruijter et al. [46] demonstrated that various calcium phosphate ceramic coatings modulated integrin expression patterns resulting in different proliferation rates of osteoblast-like cells.

Our migration assay revealed that EMD attracted more PDLs than nano-HA paste. The findings on EMD were corroborated by Chong et al. [38], who described increased migration of PDLs towards EMD. This effect might be induced by small amounts of contaminating protein within the EMD preparation that act chemotactic. While amelogenins comprise almost 90% of the proteins in EMD, the non-amelogenins include several different types of proteins, such as tuftelin, ameloblastin, enamelin, and enamel proteases [47]. However, the precise biologic mechanisms of these proteins remain unclear. Recently, it was suggested that some EMD components have growth factor-like functions [47] and act as bone morphogenetic protein-like molecules [48]. Migration towards nano-HA paste in our study was probably mediated by a stream of nanoparticles through the filter of the Boyden chamber. A possible explanation for enhanced cell migration might be the influence of calcium (Ca^{2+}) ions released by nanoparticles that directly acted on integrins, as these receptors are metalloproteins, whose activation depends on the complexation of Mg^{2+}- and Ca^{2+}-ions [49]. These divalent cations bind to the integrin subunit I and coordinate the association of integrin $\alpha\beta$-dimers with their cognate ligands. Altering the balance between Ca^{2+} and Mg^{2+} might directly affect the adhesive properties of integrin receptors, which results in altered cellular adhesion and migration.

As previously described, the dominant types of integrins found on PDL fibroblasts are β_1, α_2, α_5, and $\alpha_v\beta_3$ [50]. In accordance to these findings, we detected a prevalent surface expression of $\alpha_5\beta_1$ integrin on PDL fibroblasts. FAK, as a specific key component of signal transduction pathways triggered by integrins [51, 52] was analyzed by Western blot. Aggregation of integrins and the cytoskeletal proteins tensin, paxillin, and talin are proposed to induce FAK activation and autophosphorylation by integrins in cell adhesion [51]. As the phosphorylation and thereby activation of FAK at tyrosine residue 397 was clearly demonstrated for nano-HA and EMD, it can be concluded that these reagents cause an increase in integrin signaling. This is succeeded by increased cellular adhesion, migration, and proliferation, which we observed in our assays.

Numerous in vitro approaches described specific modifications of gingival, bone, and other mesenchymal cells in

the presence of various calcium phosphate materials [53, 54]. However, in this context, it has to be pointed out that differences in the physicochemical and structural characteristics between the novel nano-HA paste and the various forms of HA used in the past can lead to differences in the regenerative and osteoconductive properties. Material properties like porosity, surface geometry, and surface chemistry play a determinant role in osteoconductive capacities of a graft [55]. Alliot-Licht et al. [56] evaluated cellular events in PDL fibroblasts occurring in the presence of HA with a particle size <20 μm. They found that increased protein synthesis, decreased proliferation, and alkaline phosphatase activity occurred on PDLs in the presence of HA particles. Our results were corroborated by Sun et al. [24], who demonstrated that nanophase HA can promote PDL fibroblast proliferation and osteogenic differentiation compared with dense-HA. Furthermore, they reported that the increased proliferation capability of PDL fibroblasts under the influence of nanometer HA indicated a better compatibility and dissolvability of nanometer HA as compared with dense-HA. These findings were supported by Zhang et al. [25], who found that 1% nanohydroxyapatite/chitosan scaffolds enhanced proliferation of human PDLs as compared with pure chitosan scaffolds. A recent study demonstrated the ability of nanocrystalline hydroxyapatite embedded in a matrix of silica gel to promote adhesion and proliferation of PDL fibroblasts [44]. However, results obtained by our in vitro experimental model cannot recreate the complex interactions of cells in vivo. Thus, the concentration of nanostructured HA paste and EMD used in the present study and the way of dilution are different from the clinical setting.

In sum, our findings present evidence that EMD and nano-HA paste both mediate their beneficial effects via two different modes of action and therefore have different characteristics. EMD exhibited a pronounced chemotactic effect once applied in solution, and nano-HA supported cellular adhesion in its solid state, providing a basis for PDL fibroblasts to settle down. This implicates a potential synergy between both materials and a putative beneficial effect for the wound healing of patients if applied in combination. Further studies will reveal if these findings can be transferred to a clinical setting.

Conflict of interest No benefit of any kind will be received either directly or indirectly by the authors.

References

1. Pietruska MD (2001) A comparative study on the use of Bio-Oss® and enamel matrix derivative (Emdogain®) in the treatment of periodontal bone defects. Eur J Oral Sci 109:178–181
2. Vouros I, Aristodimou E, Konstantinidis A (2004) Guided tissue regeneration in intrabony periodontal defects following treatment with two bioabsorbable membranes in combination with bovine mineral graft. A clinical and radiographic study. J Clin Periodontol 31:908–917
3. Sculean A, Chiantella GC, Windisch P, Arweiler NB, Brecx M, Gera I (2005) Healing of intra-bony defects following treatment with a composite bovine-derived xenograft (Bio-Oss Collagen) in combination with a collagen membrane (Bio-Gide PERIO). J Clin Periodontol 32:720–724
4. Sculean A, Stavropoulos A, Berakdar M, Windisch P, Karring T, Brecx M (2005) Formation of human cementum following different modalities of regenerative therapy. Clin Oral Investig 9:58–64
5. Aspriello SD, Ferrante L, Rubini C, Piemontese M (2011) Comparative study of DFDBA in combination with enamel matrix derivative versus DFDBA alone for treatment of periodontal intrabony defects at 12 months post-surgery. Clin Oral Investig 15:225–232
6. Tonetti M, Pini-Prato G, Cortellini P (1996) Factors affecting the healing response of intrabony defects following guided tissue regeneration and access flap surgery. J Clin Periodontol 23:548–556
7. DeSanctis M, Clauser C, Zucchelli G (1996) Bacterial colonization of resorbable barrier materials and periodontal regeneration. J Periodontol 67:1193–1200
8. Park JS, Suh JJ, Choi SH, Moon IS, Cho KS, Kim CK, Chai JK (2001) Effects of pretreatment clinical parameters on bioactive glass implantation in intrabony periodontal defects. J Periodontol 72:730–740
9. Trombelli L, Heitz-Mayfield L, Needleman I, Moles D, Scabbia A (2002) A systematic review of graft materials and biological agents for periodontal intraosseous defects. J Clin Periodontol 29:117–135
10. Stahl SS, Froum SJ, Tarnow D (1990) Human clinical and histologic responses to the placement of HTR polymer particles in 11 intrabony lesions. J Periodontol 61:269–274
11. Nevins ML, Camelo M, Nevins M, King CJ, Oringer RJ, Schenk RK, Fiorellini JP (2000) Human histologic evaluation of bioactive ceramic in the treatment of periodontal osseous defects. Int J Periodontics Restor Dent 20:458–467
12. Bezrukov VM, Grigor'iants LA, Zuev VP, Pankratov AS (1998) The surgical treatment of jaw cysts using hydroxyapatite with an ultrahigh degree of dispersity. Stomatologiia Mosk 77:31–35
13. Thorwarth M, Schultze-Mosgau S, Kessler P, Wiltfang J, Schlegel KA (2005) Bone regeneration in osseous defects using a resorbable nanoparticular hydroxyapatite. J Oral Maxillofac Surg 63:1626–1633
14. Huber FX, Mcarthur N, Hillmeier J, Kock HJ, Baier M, Diwo M, Berger I, Meeder PJ (2006) Void filling of tibia compression fracture zones using a novel resorbable nanocrystalline hydroxyapatite paste in combination with a hydroxyapatite ceramic core: first clinical results. Arch Orthop Trauma Surg 126:533–540
15. Grigor'ian AS, Grigor'iants LA, Podoinikova MN (2000) A comparative analysis of the efficacy of different types of filling materials in the surgical elimination of tooth perforations (experimental morphological research). Stomatologiia Mosk 79:9–12
16. Gerlach KL, Niehues D (2007) Treatment of jaw cysts with a new kind of nanoparticular hydroxylapatite. Mund Kiefer Gesichtschir 11:131–137
17. Schwarz F, Bieling K, Latz T, Nuesry E, Becker J (2006) Healing of intrabony peri-implantitis defects following application of a nanocrystalline hydroxyapatite (OstimTM) or a bovine-derived xenograft (Bio-OssTM) in combination with a collagen membrane (Bio-GideTM). A case series. J Clin Periodontol 33:491–499

18. Kasaj A, Röhrig B, Zafiropoulos GG, Willershausen B (2008) Clinical evaluation of nanocrystalline hydroxyapatite paste in the treatment of human periodontal bony defects—a randomized controlled clinical trial: 6-month results. J Periodontol 79:394–400
19. Heinz B, Kasaj A, Teich M, Jepsen S (2010) Clinical effects of nanocrystalline hydroxyapatite paste in the treatment of intrabony periodontal defects: a randomized controlled clinical study. Clin Oral Investig 14:525–531
20. Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R (2000) Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. J Biomed Mater Res 51:475–483
21. Chris Arts JJ, Verdonschot N, Schreurs BW, Buma P (2006) The use of a bioresorbable nano-crystalline hydroxyapatite paste in acetabular bone impaction grafting. Biomaterials 27:1110–1118
22. Balasundaram G, Sato M, Webster TJ (2006) Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. Biomaterials 27:2798–2805
23. Pezzatini S, Solito R, Morbidelli L, Lamponi S, Boanini E, Bigi A, Ziche M (2006) The effect of hydroxyapatite nanocrystals on microvascular endothelial cell viability and functions. J Biomed Mater Res A 76:656–663
24. Sun W, Chu C, Wang J, Zhao H (2007) Comparison of periodontal ligament cells responses to dense and nanophase hydroxyapatite. J Mater Sci Mater Med 18:677–683
25. Zhang YF, Cheng XR, Chen Y, Shi B, Chen XH, Xu DX, Ke J (2007) Three- dimensional nanohydroxyapatite/chitosan scaffolds as potential tissue engineered periodontal tissue. J Biomater Appl 21:333–349
26. Ivanovski S, Haase HR, Bartold PM (2001) Isolation and characterization of fibroblasts derived from regenerating human periodontal defects. Arch Oral Biol 72:324–330
27. Rauschmann MA, Wichelhaus TA, Stirnal V, Dingeldein E, Zichner L, Schnettler R, Alt V (2005) Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials 26:2677–2684
28. Gestrelius S, Sp L, Hammarström L (2000) Emdogain—periodontal regeneration based on biomimicry. Clin Oral Investig 4:120–125
29. Ponik SM, Pavalko FM (2004) Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE2 release in MC3T3-E1 osteoblasts. J Appl Physiol 97:135–142
30. Stein RC, Waterfield M (2000) PI3-kinase inhibition: a target for drug development? Mol Med Today 6:347–357
31. Ferraz MP, Mateus AY, Sousa JC, Monteiro FJ (2007) Nanohydroxyapatite microspheres as delivery system for antibiotics: release kinetics, antimicrobial activity, and interaction with osteoblasts. J Biomed Mater Res A 81:994–1004
32. Gestrelius S, Andersson C, Lidström D, Hammarström L, Somerman M (1997) In vitro studies on periodontal ligament cells and enamel matrix derivative. J Clin Periodontol 24:685–692
33. Schwarz F, Rothamel D, Herten M, Sculean A, Scherbaum W, Becker J (2004) Effect of enamel matrix protein derivative on the attachment, proliferation, and viability of human $SaOs_2$ osteoblasts on titanium implants. Clin Oral Investig 8:165–171
34. Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL (2002) Amelogenin is a cell adhesion protein. J Dent Res 81:497–500
35. Fu Q, Zhou N, Huang W, Rahaman MN, Wang D, Zhang L, Li H (2008) In vitro study on different cell response to spherical hydroxyapatite nanoparticles. J Biomater Appl 23:37–50
36. Mateus AY, Barrias CC, Ribeiro C, Ferraz MP, Monteiro FJ (2008) Comparative study of nanohydroxyapatite microspheres for medical applications. J Biomed Mater Res A 86:483–493
37. Webster TJ, Schadler LS, Siegel RW, Bizios R (2001) Mechanisms of enhanced osteoblast adhesion on nanophase alumina involve vitronection. Tissue Eng 7:291–301
38. Chong CH, Carnes DL, Moritz AJ, Oates T, Ryu OH, Simmer J, Cochran DL (2006) Human periodontal fibroblast response to enamel matrix derivative, amelogenin, and platelet-derived growth factor-BB. J Periodontol 77:1242–1252
39. Kasaj A, Willershausen B, Reichert C, Röhrig B, Smeets R, Schmidt M (2008) Ability of nanocrystalline hydroxyapatite paste to promote human periodontal ligament cell proliferation. J Oral Sci 50:279–285
40. Zou JX, Wang B, Kalo MS, Zisch AH, Pasquale EB, Ruoslahti E (1999) An Eph receptor regulates integrin activity through R-Ras. Proc Natl Acad Sci USA 96:13813–13818
41. Burbach BJ, Medeiros RB, Mueller KL, Shimizu Y (2007) T-cell receptor signaling to integrins. Immunol Rev 218:65–81
42. Hatakeyama J, Hatakeyama Y, Takahashi I, Suzuki O, Sasano Y (2007) Proliferation and adhesion of periodontal ligament cells on synthetic biominerals. Oral Dis 13:500–506
43. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM (1996) Integrins can collaborate with growth factors for phosphorylation of receptor tyrosin kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol 135:1633–1642
44. Kasaj A, Willershausen B, Reichert C, Gortan-Kasaj A, Zafiropoulos GG, Schmidt M (2008) Human periodontal fibroblast response to a nanostructured hydroxyapatite bone replacement graft in vitro. Arch Oral Biol 53:683–689
45. Garcia AJ (2005) Get a grip: integrins in cell-biomaterial interactions. Biomaterials 26:7525–7529
46. de Ruijter JE, ter Brugge PJ, Dieudonné SC, van Vliet SJ, Torensma R, Jansen JA (2001) Analysis of integrin expression in U2OS cells cultured on various calcium phosphate ceramic substrates. Tissue Eng 7:279–289
47. Zeichner-David M, Chen LS, Hsu Z, Reyna J, Caton J, Bringas P (2006) Amelogenin and ameloblastin show growth-factor like activity in periodontal ligament cells. Eur J Oral Sci 114:244–253
48. Suzuki S, Nagano T, Yamakoshi Y, Gomi K, Arai T, Fukae M, Katagiri T, Oida S (2005) Enamel matrix derivative stimulates signal transduction of BMP and TGF-beta. J Dent Res 84:510–514
49. Leitinger B, McDowall A, Stanley P, Hogg N (2000) The regulation of integrin function by Ca(2+). Biochim Biophys Acta 1498:91–98
50. Van der Pauw MTM, Everts V, Beertsen W (2002) Expression of integrins by human periodontal ligament and gingival fibroblasts and their involvement in fibroblast adhesion to enamel matrix-derived proteins. J Periodontal Res 37:317–323
51. Guan JL (1997) Focal adhesion kinase in integrin signaling. Matrix Biol 16:195–200
52. Zamir E, Geiger B (2001) Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci 114:3583–3590
53. Sautier JM, Nefussi JR, Boulekbache H, Forest N (1990) In vitro bone formation on coral granules. In Vitro Cell Dev Biol 26:1079–1085
54. Alliot-Licht B, Gregoire M, Orly I, Menanteau J (1991) Cellular activity of osteoblasts in the presence of hydroxyapatite: an *in vitro* experiment. Biomaterials 12:752–756
55. Bauer TW, Muschler GF (2000) Bone graft materials. An overview of the basic science. Clin Orthop Relat Res 371:10–27
56. Alliot-Licht B, Delange GL, Gregoire M (1997) Effects of hydroxyapatite particles on periodontal ligament fibroblast-like cell behavior. J Periodontol 68:158–165