

## Ability of nanocrystalline hydroxyapatite paste to promote human periodontal ligament cell proliferation

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**Abstract:** Recent studies indicate that nanocrystalline hydroxyapatite (nano-HA) paste represents a promising class of bone graft substitute. However, the underlying molecular mechanisms of nano-HA function have not yet been determined. This study was conducted to investigate the proliferation of human periodontal ligament (PDL) cells cultured in the presence of nano-HA paste and to characterize associated changes in intracellular signaling pathways. Cultured PDL cells were stimulated with nano-HA paste and enamel matrix derivative (EMD) in a soluble form. Proliferation of PDL cells was determined by incorporation of bromodeoxyuridine (BrdU) in the DNA of proliferating cells. In order to understand the signaling mechanisms underlying the increased cell proliferation of PDL cells exposed to nano-HA, the phosphorylation status of the serine/threonine protein kinase Akt, of the signal regulated kinases ERK 1/2 and of the epidermal growth factor receptor (EGFR) was analyzed by Western blotting using phospho-specific antibodies. Nano-HA paste showed two-fold less proliferation potential than EMD, but both substrates increased the proliferation rate significantly ( $P < 0.05$ ) as compared with the negative control. The increased proliferation rate of PDL cells in the presence of nano-

HA paste was mechanistically linked to activation of the epidermal growth factor receptor (EGFR) and its downstream targets ERK1/2 and Akt. In conclusion, our findings suggest that nano-HA paste is a stimulator of cell proliferation, possibly contributing to the main processes of periodontal tissue regeneration. (J. Oral Sci. 50, 279-285, 2008)

Keywords: periodontal ligament; cell proliferation; periodontal regeneration; hydroxyapatite.

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### Introduction

Reconstruction of tooth supporting structures lost because of inflammatory periodontal disease is one of the ultimate goals of periodontal treatment. In order to promote regeneration of the periodontium, appropriate positioning of cells capable of synthesizing collagen, cementum and bone is required. In this context, the activities of cells derived from the periodontal ligament (PDL) play an important role (1,2). The PDL contains several cell populations including fibroblasts, cementoblasts, osteoblastic and osteoclastic cells and mesenchymal cells. Thus, the complex process of periodontal regeneration requires the recruitment of PDL cells, and their proliferation, differentiation and colonization of the wound area.

One approach to achieve periodontal regeneration is the use of bone replacement graft techniques, which are based on the concept of filling the intrabony defect with a number of grafting materials, including autografts, allografts, xenografts and alloplastic materials. Calcium-

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phosphate-based inorganics, especially hydroxyapatite (HA), are particularly useful alternatives to autogenous bone grafts in orthopedic, dental and maxillofacial applications, due to their chemical and structural similarity to the mineral component of bone (3-5). Although these materials are highly biocompatible, one of the problems related to the use of mineral-containing biomaterials is the release of crystals or agglomerates that can impair cell activity and hinder regeneration processes. In this context, the use of hydroxyapatite-based biomaterials for regenerative periodontal treatment has been shown to significantly improve probing depths and clinical attachment levels (6,7). However, histological analysis has revealed not only an unpredictable amount of periodontal regeneration after HA grafting of intrabony periodontal defects (8,9). As natural bone comprises nanoscale features, it is believed that nanostructured HA can improve the properties of synthetic bone, due to its higher surface area.

Recently, a fully synthetic nanocrystalline hydroxyapatite (nano-HA) paste (Ostim<sup>®</sup>, Heraeus Kulzer, Hanau, Germany) containing about 65% water and 35% nanostructured apatite particles, has been introduced for augmentation procedures in osseous defects (10,11). Advantages of such a nanostructured material in comparison to bulk material are its close contact with surrounding tissues, quick resorption characteristics and a high number of molecules on its surface. It was found that undisturbed osseous-integration and complete resorption of nano-HA paste occurs within 12 weeks (12). The nano-HA paste has already been used not only for the treatment of various types of metaphyseal fractures such as the calcaneus and tibia in orthopaedic surgery (13), but also for tooth perforations (14), jaw cysts (15) and peri-implantitis lesions (16). However, there is no information available about the biologic response of PDL cells to application of nano-HA paste. Since interactions between periodontal ligament (PDL) cells and bone graft materials are important for periodontal wound regeneration, we investigated the interactions of cultured human PDL fibroblasts with a novel nanostructured hydroxyapatite paste. Comparison of the results with those for enamel matrix derivative (EMD) allowed us to determine possible similarities or differences in the mechanisms mediated by both materials.

The purpose of the present study was to investigate the proliferation of PDL cells cultured in the presence of nano-HA paste and to clarify the accompanying changes in cellular signaling pathways.

## Materials and Methods

### Reagents

The nano-HA paste (Ostim<sup>®</sup>, Heraeus Kulzer) consists

of a suspension of pure nanocrystalline HA in water prepared by a wet chemical reaction. After preparation of the paste, the HA content is 35%. For the present cell proliferation experiments, nano-HA paste was dissolved in phosphate-buffered saline (PBS, pH 5.2) (Gibco Laboratories, Grand Island, NY, USA) to a final concentration of 100 µg/ml.

To create a viscous solution required for the experiment, the lyophilized EMD (Emdogain<sup>®</sup>, Straumann, Basel, Switzerland) was mixed with 3 ml of a 7.5% acetic acid solution to achieve a stock solution of 10 mg/ml. To obtain the proper concentration, 100 µl of this EMD solution was mixed with 9.9 ml Dulbeccos's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (PAA, Pasching, Austria) to create a final solution of 100 µg/ml with a pH of 6.8-7.2.

Recombinant human EGF and recombinant human PDGF-BB were purchased from Sigma (St. Louis, MO, USA) and reconstituted in phosphate-buffered saline (PBS) before use. Bovine serum albumin (BSA) was purchased from Sigma, and used without further purification.

### Cell cultures and media

Human PDL cells were harvested from human periodontal tissues isolated from third molars extracted for orthodontic reasons in three systemically healthy adult patients. Prior to extraction, patients were informed about the study and agreed to experimental use of their extracted teeth. The study was conducted in accordance with the second Helsinki Declaration. Under sterile conditions, the PDL tissue fragments were mechanically removed by scraping the middle third of the root surface with a sharp blade. Tissue explants were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 1% penicillin/streptomycin (Invitrogen), 1% fungizone (Sigma) and 10% FBS (PAA). 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<sub>2</sub> and 95% air. Tissue culture medium was changed every 2 days until confluence (approximately 7×10<sup>4</sup> cells/cm<sup>2</sup>) was reached, and the cells were passaged at a 1:2 split ratio following trypsinization with 0.05% trypsin (Invitrogen). Cell cultures were also tested regularly for mycoplasma. The cells were used for the experiments between the fourth and ninth passages. All experiments were performed in triplicate using cells prepared from three different donors.

### Cell proliferation assay

Proliferation rate was determined by overnight BrdU incorporation using a cell proliferation ELISA (Roche,

Basel, Switzerland) in accordance with the manufacturer's guidelines. In brief, PDL fibroblasts were plated on 35-mm Petri dishes coated with various compounds at  $5 \times 10^3$  cells/Petri dish in DMEM with 10% FBS. The culture plates were incubated for 24 h with BrdU at 37°C in a 5% CO<sub>2</sub> atmosphere. Next day, the cells were harvested and BrdU incorporation measured using the BrdU ELISA. Each experiment was performed in triplicate for each experimental group (BSA, nano-HA, EMD, EGF and PDGF-BB).

### <sup>125</sup>I-EGF binding assay

The capacity of the PDL fibroblasts to bind EGF was analysed using a <sup>125</sup>I-EGF binding assay. To examine altered binding of EGF to its receptor, EGFR, the cells were incubated with 1 ng ml<sup>-1</sup> <sup>125</sup>I-EGF in DMEM plus 0.2% BSA (Sigma) for 2 h at 4°C in the presence or absence of 1,000 ng ml<sup>-1</sup> nano-HA, EMD or EGF. Subsequently, the cells were thoroughly washed, lysed, and remaining cell-associated radiation was measured in a gamma-counter (1470 Wizard, Perkin Elmer, Boston, USA). Experiments were performed three times, and mean values were calculated.

### Western blotting

For detection of proteins in western blots, monoclonal mouse anti-phospho-Erk (E-4), anti-pY-EGFR 1173, as well as polyclonal rabbit anti-Erk2 (C-14) and anti-EGFR (1005) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Further reagents used were rabbit monoclonal anti-Akt and anti-pS-Akt (Ser473) antibodies (Cell Signalling Technology, Beverly, MA, USA).

Protein samples were analyzed by SDS-PAGE using an XCell SureLock Mini-Cell (Invitrogen) 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 volts in accordance with the manufacturer's guidelines. After electrophoresis, proteins were blotted onto a PVDF membrane and incubated for at least one hour in blocking buffer (5% BSA and 1% Tween-20 in Tris-buffered saline (TBS) (Sigma). The membranes were incubated overnight with appropriate dilutions of primary antibody in blocking buffer. The next day, the membranes were washed and incubated for one hour 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). After additional washing steps, antibody complexes were visualized on film by ImmunoStar AP substrate (Bio-Rad, Hercules, USA). Western blot results were quantified by densitometry (Software ImageJ, NIH, USA).

### Data analysis

All statistical analyses were performed using statistical software (SPSS, 12.0 for Windows, Chicago, IL, USA). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Scheffe's post-hoc test. Differences were considered significant at  $P < 0.05$ .

### Results

In order to elucidate the role of nano-HA paste in periodontal regeneration, PDL cell proliferation was assessed and compared to the responses induced by nano-HA paste and EMD. Our results showed that all of the tested substrates significantly ( $P < 0.05$ ) enhanced the proliferation of PDL fibroblasts in comparison to the negative control (Fig. 1). EGF and PDGF-BB were used to achieve a maximum of cellular proliferation and acted as a positive control in this experiment. Nano-HA paste yielded a significantly ( $P = 0.001$ ) lower cell proliferation rate than EMD, but both substrates increased the proliferation rate significantly ( $P < 0.05$ ) relative to the negative control bovine serum albumin (BSA). EGF and PDGF-BB were the most potent mitogens for PDL cells and yielded a significantly ( $P < 0.001$ ) higher cell proliferation rate than EMD and nano-HA.

In order to test the hypothesis that both substrates affect ligand binding to receptor tyrosine kinases such as the epidermal growth factor receptor, and thereby alter PDL

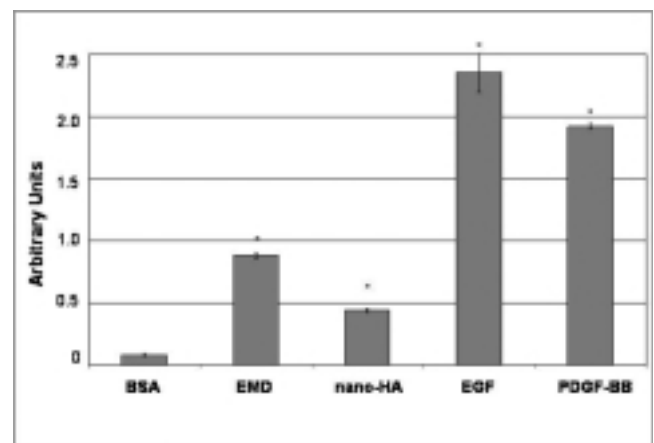


Fig. 1 Effect of nano-HA paste, EMD, EGF and PDGF-BB on PDL cell proliferation. Cells were plated on 35-mm cell culture dishes coated with the various compounds at  $5 \times 10^3$  cells per dish in DMEM with 10% FBS. Proliferation was determined by BrdU incorporation. Error bars represent the standard deviation of the mean from three separate experiments. \*Significantly different from negative control ( $P < 0.05$ ).

proliferation, we examined if nano-HA paste or EMD alter binding of radioactive labelled  $^{125}\text{I}$ -EGF to the EGFR in PDL cells. Both compounds were added to the culture medium along with  $1\text{ ng ml}^{-1}$  of  $^{125}\text{I}$ -EGF. Addition of  $1,000\text{ ng ml}^{-1}$  served as a quality control for the assay, as a 1,000-fold excess of cold EGF should effectively compete out  $^{125}\text{I}$ -EGF from EGFR binding. This was indeed the case, which verified that the assay worked ( $P < 0.001$ ). However,

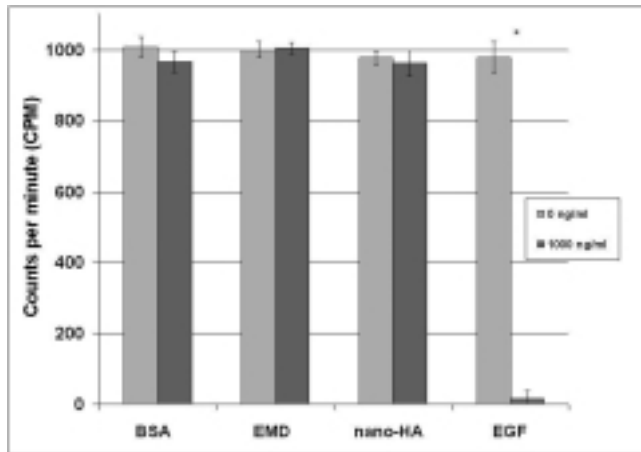


Fig. 2 Effect of nano-HA paste, EMD and EGF on subsequent  $^{125}\text{I}$ -EGF binding. Cells were incubated with  $1\text{ ng ml}^{-1}$   $^{125}\text{I}$ -EGF in DMEM plus 0.2% BSA for 2 h at  $4^\circ\text{C}$  in the presence or absence of  $1,000\text{ ng ml}^{-1}$  nano-HA, EMD or EGF. All data reported represent the standard deviation of the mean from three separate experiments. \*Significant at  $P < 0.001$ .

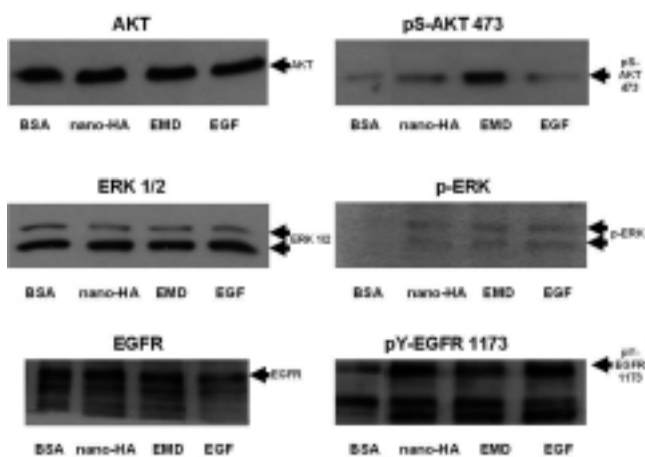


Fig. 3A Western blot analysis of total and phosphorylated serine/threonine protein kinase Akt, extracellular signal-regulated kinase ERK 1/2, and epidermal growth factor receptor (EGFR) after stimulation with nano-HA paste, EMD or EGF. Representative Western blots for Akt, ERK 1/2, and EGFR are shown.

binding of EGF to EGFR was not affected by nano-HA or EMD (Fig. 2;  $P > 0.05$ ).

Phosphorylation of Akt/PKB in PDL cells was detected by Western blotting using an antibody against Akt/PKB phosphorylated at the Ser473 residue. EGF did not affect receptor phosphorylation as compared with the negative control, whereas nano-HA paste and EMD clearly induced activation of Akt/PKB (Figs. 3A and B).

Phosphorylation of ERK 1/2 in PDL cells was detected by Western blotting using an antibody against phosphorylated Erk 1/2. We observed a significant increase in the amount of pERK activity in response to nano-HA, EMD and EGF. In order to determine if endogenous EGFR was activated in our PDL cells, we performed Western blot analysis of PDL cells in the presence of BSA, nano-HA,

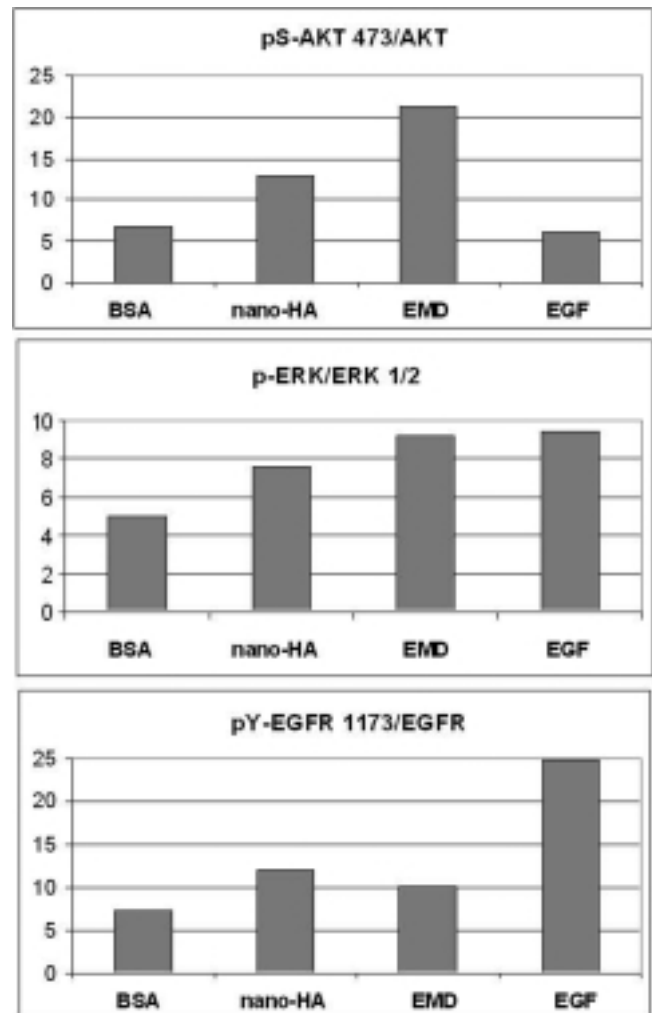


Fig. 3B The graphs show Western blot results quantified by densitometry, and represent one of the triplicate experiments. Protein phosphorylation is calculated as the ratio of the phosphorylated vs nonphosphorylated forms.



EMD or EGF, and blotted for activated epidermal growth factor receptor (pY-EGFR 1173). Upon activation of EGFR the receptor is phosphorylated at tyrosine 1173. Nano-HA and EMD induced activation of the EGFR, where the phosphorylation level of cells stimulated by nano-HA was even higher in comparison to EMD (Fig. 3A and B). A significant increase in the amount of phosphorylated EGFR was observed after exposure to EGF.

## Discussion

Over the last few decades, a wide range of procedures have been proposed for the treatment of periodontal disease to promote bone regeneration in intraosseous defects, among them the use of bone replacement graft materials. These materials should support attachment and proliferation of PDL cells, which are involved in the repair of damaged periodontal tissues. Recent studies have shown that nanocrystalline HA represents a promising class of bone graft substitute (11,17). Our results revealed that PDL cells were able to proliferate in the presence of nano-HA paste. The examined PDL cells showed a proliferative response to EMD, as has already been documented for this cell type (18,19), being double the response to nano-HA paste. Our findings that EGF and PDGF-BB increased the proliferation of PDL cells are consistent with previous data (20,21).

The <sup>125</sup>I-EGF binding assay revealed that there was no competitive binding between EGF, nano-HA paste and EMD, indicating that EGFR is not the binding site for nano-HA or EMD. Our findings indicating that the specific binding of <sup>125</sup>I-EGF to PDL cells was not affected by treatment with EMD are in agreement with a previous study (22). The enhanced effects of nano-HA paste and EMD might be linked via integrin-receptor tyrosine kinase cross-talk. Indeed, there is mounting evidence that integrins transduce signaling pathways via phosphorylation of focal adhesion kinases (FAKs), leading to ERK 1/2 activation (23). Although the effect of nano-HA paste on PDL cells has not been investigated, numerous *in vitro* approaches have described specific modifications of gingival, bone and other mesenchymal cells in the presence of various calcium phosphate materials (24,25). Alliot-Licht et al. (26) evaluated cellular events in PDL fibroblasts occurring in the presence of HA with a particle size of <20 µm. They found that increased protein synthesis, decreased proliferation and alkaline phosphatase activity occurred on PDL cells in the presence of HA particles. Our results were corroborated by Sun et al. (27), who demonstrated that nanophase HA can promote PDL fibroblast proliferation and osteogenic differentiation in comparison with dense-HA. Furthermore, they reported that the increased

proliferation capability of PDL fibroblasts under the influence of nanometer-order HA indicated that the latter had better compatibility and dissolvability than dense HA. Recent research has shown that synthetic nanostructured HA enhances osteoblast functions (28) and has higher biocompatibility for microvascular endothelium (29). Furthermore, it has been shown that proteins interact differently with nanophase materials than with conventional ceramics having similar chemical properties (30).

The present study demonstrated that the increased proliferation rate of PDL cells in the presence of nano-HA paste was mechanistically linked to activation of the epidermal growth factor receptor (EGFR) and its downstream targets ERK1/2 and Akt. Our observations that the mitogenic response of human PDL cells was accompanied by autophosphorylation of EGFR and the subsequent activation of ERK 1/2 are in agreement with previous findings (20). Since ERK 1/2 are also reported to be involved in growth factor production (31), activation of this pathway may play a role in regulation of the indirect effect of nano-HA. Another mechanism, which enhances cell proliferation and inhibits apoptosis, is activation of the Akt pathway (32), which was clearly demonstrated for nano-HA paste and EMD in the present study.

In conclusion, the present findings indicate that nano-HA paste is a stimulator of cell proliferation. This mitogenic effect of nano-HA paste was mediated by EGFR, followed by activation of ERK1/2 and Akt. In a clinical setting, these pathways would be expected to act in concert at sites of periodontal regeneration.

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