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Comparison of periodontal ligament cells responses to dense and nanophase hydroxyapatite

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Abstract Hydroxyapatite, a synthetic calcium phosphate ceramic, is used as a biomaterial for the restoration of human hard tissue as well as in techniques which aim to regenerate periodontal tissues. Generally, hydroxyapatite is believed to have osteoconductive effects and to be non-bioresorbable but not to induce to periodontal tissue regeneration. No report has been found on responses of periodontal ligament cells (PDLC), the main contributor to periodontal tissue regeneration, to nanoparticles of hydroxyapatite. The objective of this study was to investigate the possible effects of nanophase powder of hydroxyapatite on proliferation of periodontal ligament cells. Using a sol-gel method, the nanophase hydroxyapatite powders were fabricated. These powders were proved to comprise nanoparticles by transmission electron microscope examination. The primary periodontal ligament cells were cultured on dense particle hydroxyapatite and nanometer particle hydroxyapatite. The effects on proliferation of periodontal ligament cells on dense and nanoparticle hydroxyapatite were examined in vitro using a methyl thiazolil tetracolium (MTT) test. The intercellular effects were studied with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray (EDX). In addition, the influence of the two materials on osteogenic differentiation was determined through measurement of alkaline phosphatase activity and flow cytometry. About 2, 3, and 4 days after treatment with nanoparticles of hydroxyapatite, the proliferation activity of the PDLC increased significantly compared with those proliferating on dense hydroxyapatite and of control PDLC, but no significant difference was found between the PDLC proliferation on dense hydroxyapatite and the control PDLCs. After 3 and 5 days' incubation with nanoparticles of hydroxyapatite, alkaline phosphatase activity was significantly increased as compared to PDLCs incubated with dense hydroxyapatite and control PDLCs. Intracellular engulfment was found in the cultured cells with nanophase hydroxyapatite under electron microscopy. The results suggest that nanophase hydroxyapatite can promote proliferation and osteogenic differentiation of periodontal ligament cells and further that it may be used as a bioresorbable agent in osseous restoration.

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1 Introduction

One purpose of periodontal therapy is the regeneration of tissue, which has been lost due to destructive periodontal disease. Many bone substitutes have been suggested for use in periodontal regeneration [1]. Hydroxyapatite, a synthetic calcium phosphate, is commonly used in periodontal surgery for restoration of lost periodontium. Many reports recommend this material because of its good biocompatibility and osteoconductibility [2, 3]. However, according to some studies, new periodontal regeneration has not always

been found if hydroxyapatite is used in the treatment of periodontal bone loss [4]. It is believed that the failure of regeneration is due to the property of the calcium phosphate material, which has only osteoconductive but no osteoinductive effect on periodontal osseous defects. In recent years, more and more studies have been designed to determine the effects of growth factors and biological materials on proliferation of periodontal ligament cells [5, 6]. However, when these factors, i.e. hydroxyapatite (HA), are used in the periodontal osseous destruction, new periodontal regeneration has rarely found [7]. In this study, nanophase hydroxyapatite particles were prepared using a sol–gel method and its effects on proliferation and osteogenic differentiation of periodontal ligament cells were assessed for the significance of the biomaterial for periodontal regeneration and compared with the effects of dense hydroxyapatite.

2 Materials and methods

2.1 Nanophase hydroxyapatite powders

Nanophase hydroxyapatite powders were fabricated using a sol–gel method [8]. Aqueous solution of calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$] and alcohol solution of trimethyl phosphate ($((CH_3O)_3PO)$) were used as precursors. A small amount of citric acid (about 5%), used as a chelating reagent, was added to the aqueous solution of calcium nitrate tetrahydrate. The solutions were mixed at room temperature with Ca/P in mole ratio of 5:3 and were kept at pH 7.5 by ammonium hydroxide. After 48 h reaction, the gel thus obtained was dried at 190 °C for 2 h, calcined at 600 °C for 1 h and ground into fine powders.

Dense and polycrystal hydroxyapatite, which was supplied by Longma Chemical Company Ltd. of Xinjin, Chengdu, China, was used for incubating PDLCs in another experimental group. The granularity of these particles is about 420–842 μm.

2.2 Primary cell cultures [9]

Human periodontal ligament fibroblast-like cells were obtained from root scrapings of healthy permanent teeth, which just had been surgically extracted for orthodontic reasons. Before the operation, the patients or his/her parents (in child) gave informed consent to the use of the extracted tooth/teeth. After being carefully scraped from the mid-third of the root, the soft tissue was placed into a Petri dish containing PBS solution with 200 U/ml penicillin, 200 μg/ml streptomycin sulphate to clean the specimens. Then the sample was moved into a sterilized centrifuge tube with 0.25% collagenase for 50–60 min and 0.125% trypsin for another 20–25 min. Minimal essential medium with 10% fetal calf serum was not added to the pipe until spear cells were observed under microscope. After the cell suspension was centrifuged in 1,000 rps for 10 min, the supernatant fluid was removed and deposit was added to 4 ml of minimal essential medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. These explants were seeded in culture flasks and the medium was changed every 3 days in humidified 95% oxygen, 5% CO_2 atmosphere at 37 °C. Confluent monolayers were obtained by 3 weeks and the cells were subcultured after exposure to 0.25% trypsin. Cells of the 4th passages were evaluated with anti-vimentin and anti-keratin immunohistochemistry for interstitial character. Cells from the 4th to 8th passages were retained for experiments.

2.3 Incubation of cells and materials

Cells were seeded at a concentration of 1×10^4 cells/well for 1 ml in 24-well plates. For the scanning electron microscopy, a cover glass plate (1 cm × 1 cm) was put into each well and then seeded. After 24 h, materials were added as follows: Group 1: nanophase hydroxyapatite powder (nano-HA) 1 mg/ml for 6 wells; Group 2: dense hydroxyapatite (dense-HA) 1 mg/ml for 6 wells; Group 3: medium alone as control for 6 wells.

2.4 Scanning electron microscopy, transmission electron microscopy and Energy dispersive analysis X-rays

On the 5th day of culture, the glass plates were fixed with 2.5% glutaraldehyde for 24 h before being post-fixed for 1 h with 1% osmium tetroxide. Then the samples were dehydrated with series of graded alcohol solutions, critical-point-dried and sputter coated with gold. The mounted slabs were observed with scanning electron microscopy and affiliated energy dispersive X-ray (Sirion® Field Emission SEM, Germany).

At the same time, the medium was removed and washed with PBS for three times. The cells were put into an Apendoff's tubes. After centrifuging (4000 rps, 1 min), the supernatant fluid was removed and the deposit was treated with 2.5% glutaraldehyde in cacodylate buffer for 24 h and post-fixed with 1% osmium tetroxide for 1 h. The cell pills were washed in Ca^{++}–Mg^{++}–$NaHCO_3$-free PBS, dehydrated through a

graded series of ethanol concentrations, and embedded in Epon 812. Serial ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined using transmission electron microscopy and affiliated energy dispersive X-ray (JEM-2010 EM, Japan).

2.5 Methyl thiazolil tetracolium (MTT) test

After the cells and materials were incubated in atmosphere at 37 °C and 5% CO_2 for 4 days, 100 µl MTT was added to each well and then incubation was continued for 4 h. Cell cultures were stopped before the medium was replaced with 150 µl dimethylsulfoxide (DMSO) and oscillated for 10 min. The OD_{450} value was determined with ELISA analyzer.

2.6 Alkaline phosphatase (ALP) specific activity

On the 5th, 8th day of culture, cells were rinsed twice with PBS and digested by 0.25% trypsin (AMRESCO Inc., USA). Cells were added to a solution of 10 mmol/l Tris–HCl, 5 mmol/l $MgSO_4$, 0.1% Triton X-100, 0.1% $NaNO_3$, and frozen (–20 °C) three times to disrupt cell membranes. Thawed cell suspensions were sonicated for 5 min in order to extract the enzyme. Alkaline phosphatase activity was measured in the supernatant by an established technique using *p*-nitrophenyl-phosphate (Sigma Company, USA) as a substrate at 37 °C. After 30 min, the reaction was stopped with 3 mmol/l NaOH. At the same time, standard samples and blank controls were prepared. The absorbance at 405 nm was read on a plate reader.

2.7 Flow cytometric (FCM) analysis

After 5 days of culture, confluent cultures of the cells and materials were rinsed twice with PBS and collected with a sterile scraper and suspended in separate tubes at a volume of 250 µl. The primary mouse monoclonal antibody against ALP was diluted to 1:100 in growth media. Cells were incubated with the primary antibody for 20 min at 4 °C. After washing, cells were incubated for an additional 20 min at 4 °C with the secondary antibody for ALP, FITC conjugated goat monoclonal antibody against mouse (diluted to 1:500 in growth medium). After washing, cells were suspended in 300 µl of PBS and analyzed with a flow cytometer.

2.8 Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) and post-hoc tests were performed using SPSS.

3 Results

Primary periodontal ligament cells germinated by 4 days, increased rapidly after 7 days and were confluent in about 2 weeks, taking the form of monolayer fibroblast-like cells. The characterization of these cells expressed positive stain of vimentin and negative of keratin (Fig. 1).

Using TEM investigation, many nanophase particles were observed with dimensions on average of less than 50 nm. Partial HA crystal grains could be seen to aggregate into the so-called ‘‘secondary particles’’ as the result of the spontaneous congregation (Fig. 2) and energy dispersive X-ray (EDAX) showed the features of hydroxyapatite (Fig. 3). In the EDAX spectra (Fig. 3) the elements of C, P, O and Ca are identical with qualities of hydroxyapatite and Pt and Cu come from electrical dyeing for electron microscopy.

After adding nanoparticles of hydroxyapatite, isolated cells manifested satellite morphology, displaying numerous long cytoplasmic extensions and marked nucleoli. On the 5th day after seeding, scanning electron micrograph observation of these cells showed many particle-like materials to be stuck on the surface of the cells (Fig. 4) and some were undergoing phagocytic processes in cells, in which cells many less than 1 µm particles appeared. The content of these particles

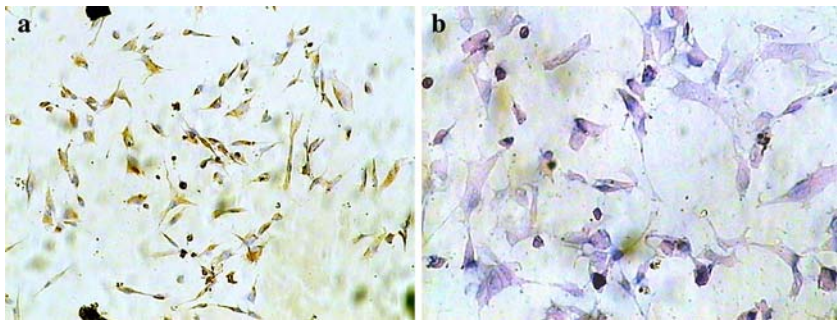


Fig. 1 Immunocytochemical staining on the primary periodontal cells (original magnification 40×). (**a**) Positive staining for vimentin on primary periodontal ligament cell (brown color in plasma). (**b**) Negative staining for keratin on same cells

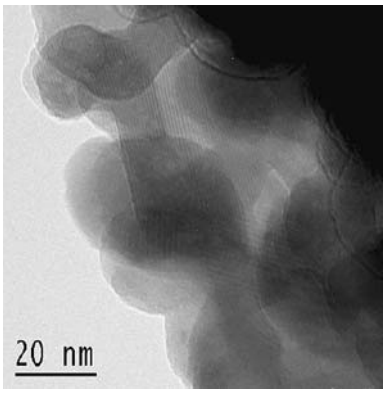


Fig. 2 The morphologies of nanometer sized HA powders in TEM and EDX spectra

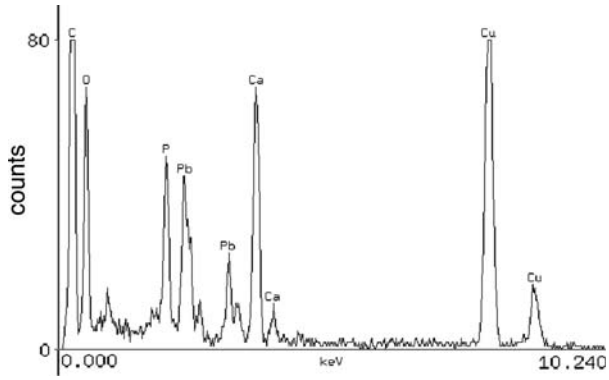


Fig. 3 EDX spectra of nanometer sized hydroxyapatite powders

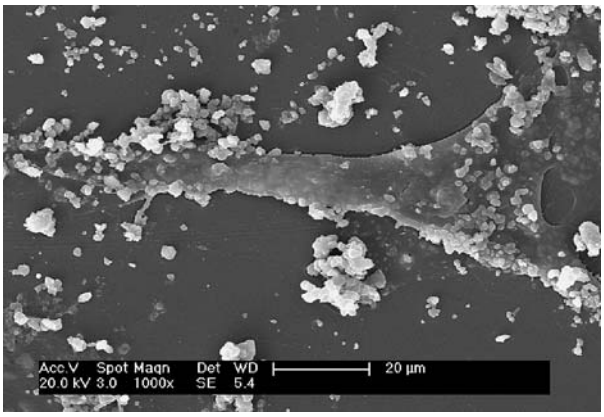


Fig. 4 Scanning electron micrograph of periodontal ligament cells in the presence of nanometer HA powders

and cell membrane are shown in Fig. 5 where obvious hydroxyapatite contents at the site of the particles could be identified and high carbon content was shown at the site of the membrane (Fig. 5). From these EDX profiles in Fig. 5(c, d), much Si can be detected. It is believed that the element Si was derived from the glass plates.

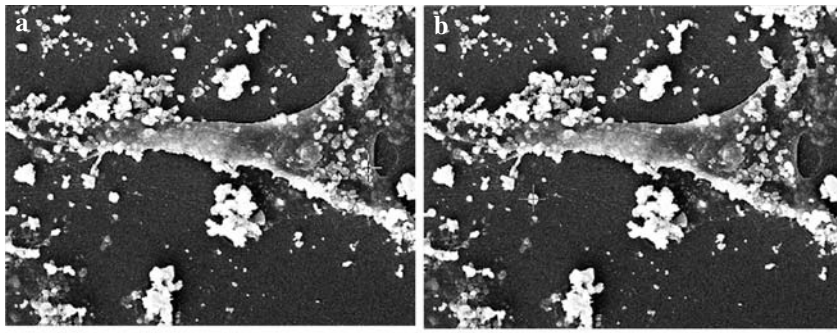
In TEM micrographs, there were a mass of gross-like particles intracellularly, the diameter of which was about 5 nm. The condition of the cells which had engulfed these particles appeared normal. Their organelles and the nuclei were clear and no prominent changes were seen. None of these fine particles were visible in cells cultured with dense hydroxyapatite as well as the control though some pieces of the materials could be seen intercellularly (Fig. 6).

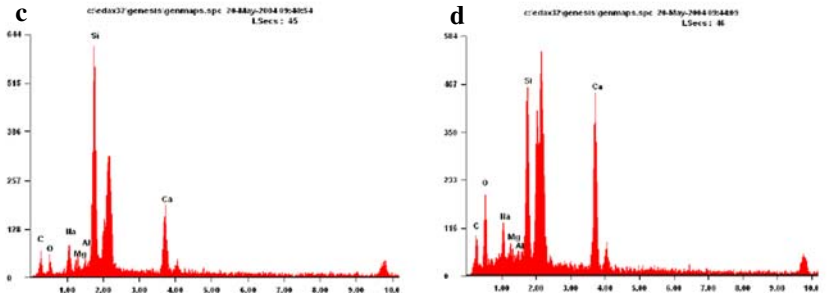
MTT testing showed that peak proliferation appeared at day 4 in nano-HA, dense-HA and control group. In the presence of nanoparticles of HA, the level of OD_{450} indicated that cell proliferation was significantly the highest (Table 1), but no statistically significant difference could be seen between dense-HA group and the control through post-hoc tests (Table 2).

Figure 7 shows the ALP activity of PDLC after being cultured in the presence of the extract from each specimen (Fig. 7). The incubation for 5 and 10 days with nano-HA demonstrated significantly greater ALP activity than the culture with the dense-HA and the control. In FCM, the expression of ALP was significantly higher in the group with nanoparticles than those in others two groups on day 5 and day 8 (Fig. 8). There were however no statistically significant differences in ALP activity between the groups of the dense-HA and the controls, in spite of the levels of the groups being lower than that of nano-HA.

4 Discussion

In the 1980s, a novel procedure was proposed in which a physical barrier was introduced by surgically placing a membrane between the connective tissue of the periodontal flap and the curetted root surface [10] to enhance periodontal regeneration. At the same time, many materials, including bioactive glass, hydroxyapatite, extracelluar matrix components and other synthetic materials, have been applied in clinical situations with a similar objective [11, 12]. Although no clear guidelines have been established for choice of the bone replacement materials, artificial bone materials should be carefully selected because for safety and efficacy reasons. However, the results of studies which showed that most artificial materials have not been found to induce cellular differentiation has lead to limited usage of these materials in periodontal regeneration. Alliot-Licht [13] prepared <20 μm hydroxyapatite powder with a mortar and sifting. After the powder was added to incubating periodontal ligament cells, the experimental group expressed increased protein synthesis and decreased ALP-specific activity. These





e

Element	Wt%	At%
C K	49.75	65.63
O K	19.78	19.59
NaK	07.32	05.04
MgK	01. 52	00.99
AlK	00.93	00.54
CaK	20.70	08.19

f

Element	Wt%	At%
C K	27.36	39.95
O K	39.05	42.81
NaK	06.08	04.64
MgK	01.60	01.16
AlK	00.48	00.32
CaK	25.42	11.12

Fig. 5 SEM and EDX analysis for specimen treated with nanophase hydroxyapatite (**a**, **c**, **e**) shows the result of SEM and EDAX analysis for nanophase hydroxyapatite that located in cells. (**b**, **d**, **f**) Shows the result of SEM and EDX analysis for nanophase hydroxyapatite that located out of cells. (**a**) SEM image of PDLC and powder of nanophase hydroxyapatite. (**b**) The image of same position but at the point (cross) of particle engulfed by the cell. (**c**) EDX spectra of the particle (labeled by cross). (**d**) EDX spectra of the point in picture **c**. (**e**) Six kinds of elements content of nanophase hydroxyapatite that located in cells, and high carbon content were shown. (**f**) Six kinds of elements content of nanophase hydroxyapatite that located out of cells

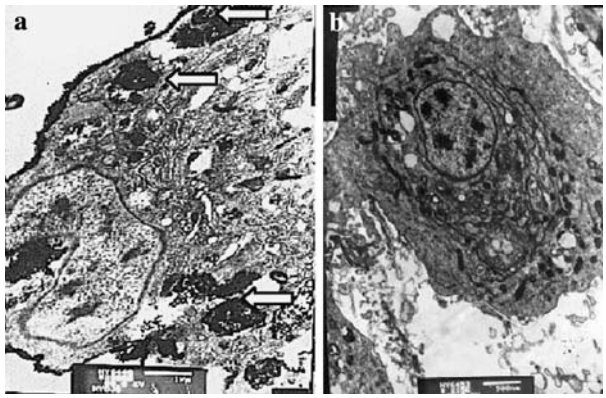


Fig. 6 TEM images of PDLC cultured with nano-HA (**a**) and dense-HA (**b**). The marker in (**a**) indicate the mass of granules in PDLC

results indicate that the HA appears to have a specific effect on osteoinduction. Besides, this study also gave us some new information concerning the role of human periodontal fibroblastic cells during repair of periodontal bone defects in the presence of HA powder.

In this study, the addition of citric acid as a chelating reagent during the sol–gel process was undertaken in order to prevent the agglomeration of hydroxyapatite powders. The influence of citric acid on the dimension of nanophase hydroxyapatite powders can be interpreted through the hydrolysis reaction of metal ions. Generally, the hydrolysis speed is higher than that of deposition, which would appear before the formation of the gel. The stable gel cannot form unless the hydrolysis and condensation processes are very slow. By the addition of citric acid as a chelating reagent, the substitution of organic macromolecule for hydration molecule can reduce the reaction speed in the sol-gel system. It is addition of the citric acid that makes the sol be the stable gel, in which the produced hydroxyapatite gel particles are difficult to aggregate and grow after the hydrolysis and condensation reactions. Consequently, the dispersible *nanometer* hydroxyapatite powder could be prepared [8].

Table 1 ANOVA analysis of OD_{450} in the cell proliferation in three groups at each time interval

	n	Nano-HA	Dense-HA	Control	*F*	*P*
2nd day	6	0.245 ± 0.273	0.119 ± 0.045	0.128 ± 0.036	28.41	0.000
3rd day	6	0.306 ± 0.013	0.185 ± 0.064	0.178 ± 0.084	8.169	0.004
4th day	6	0.689 ± 0.065	0.317 ± 0.063	0.337 ± 0.042	79.027	0.000

Table 2 Post-hoc multiple comparisons among the three groups at each time interval

		Nano-HA	Dense-HA
2nd day	Dense-HA	0.000	–
	Control	0.000	0.655
3rd day	Dense-HA	0.004	–
	Control	0.003	0.84
4th day	Dense-HA	0.000	–
	Control	0.000	0.547

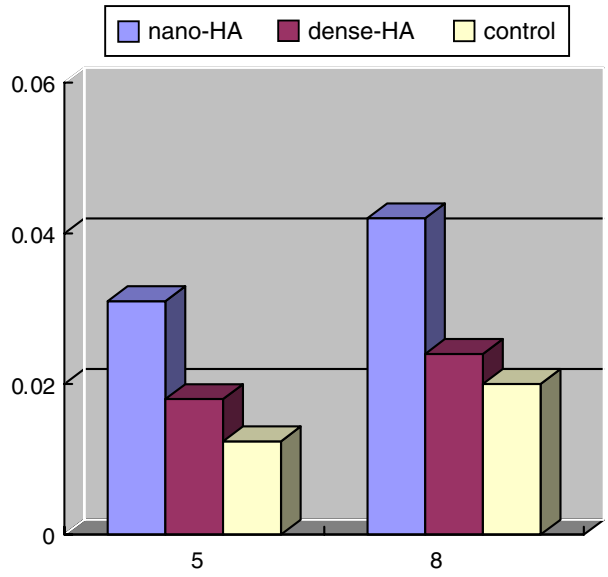


Fig. 7 The ALP activity of PDLC cultured with nanoparticles and dense particles of HA on day 5 and day 8. The incubation for 5 days and 10 days with nano-HA demonstrated significantly greater ALP activity than the culture with the dense-HA and the control

The data showed the positive proliferation activity of nanometer hydroxyapatite powder on cultured periodontal ligament fibroblasts. In the nano-HA group, the number of cells was significantly higher than that of dense-HA group after 2 days reaction of addition of the material, and it was markedly higher on the 4th day due to the logarithm phase in which the effect of nanophase material appeared to exert a significant effect. However, line increasing cell proliferation was observed in the dense-HA group and the control group but significant difference was found between the experimental group and the control groups using ANOVA. Post-hoc tests showed significant differences between the nano-HA and dense-HA group at all time intervals, but no significant differences between the dense-HA and the control on 2nd, 3rd and 4th day were noted. This indicates that nanophase hydroxyapatite has the effect of increasing proliferation of periodontal ligament cells.

When cultured with the nanometer HA, enhancement of ALP activity was obtained after 5 days of incubation, and greater decrease in its level than when cultured with dense-HA was observed on day 10. At the same time, nanometer HA stimulated the expression of ALP. Both ALP activity and ALP intracellularly are important markers of osteogenic differentiation, and ALP activity is a primary functional component of the extracellular matrix [14–16]. Our findings suggest that nanometer HA accelerates osteogenic differentiation at an early stage and promotes extracellular matrix formation in comparison with dense-HA. For these reasons, we examined the differentiation and mineralization of periodontal cells cultured with each. ALP activity is one of the representative markers of the osteoblast phenotype. It is generally accepted that increased specific activity of ALP in a population of PDLC reflects a shift to a more differentiated state. Furthermore, ALP appears to have a crucial role in the initiation of matrix mineralization as the expression of this enzyme is down-regulated after mineralization starts.

The results of TEM showed that there was an abundance of conglobulations, which were several nanometers in diameter and gathered in the PDLC cultured with the nanometer HA. The electronic density of the globose granules was obviously higher than the other intracellular ingredients. The electronic dispersive analysis of these granules showed that they were intracellular hydroxyapatite composed of calcium phosphate. It could be concluded that the nanometer HA had better biological compatibility because of the normal basic cell form preserved in the PDLC and having exerted no evident toxicity. On the other hand, there were no intracellular granules in the PDLC cultured with the dense-HA as observed through TEM; however quantities of granules existed in extracellular component. The results indicated that there were different absorbance modes between the dense-HA group and the nanometer HA and the nanometer HA was likely to be a dissolvable biological material. Granules normally have three pathways to gain

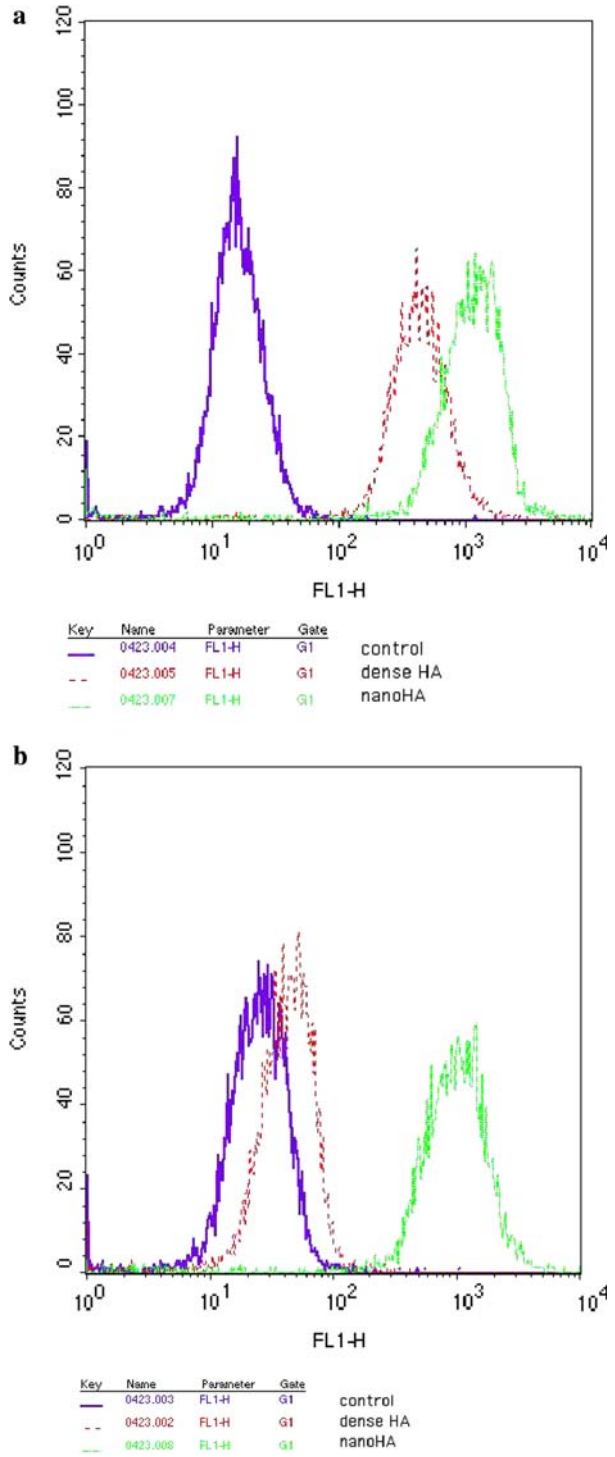


Fig. 8 FCM images of distribution of ALP positive cells in three groups on day 5 (**a**) and day 8 (**b**). The distribution of ALP positive cells cultured with nanoparticles were significantly greater than others groups on day 5 and day 8

intracellular access: pinocytosis, pinocytotic process through receptor and 'swallowing'. The modes which nanometer granules assume in media are diversiform, for example, single granules and granules conglomeration, which are several tens of nanometers in diameter. Due to the higher phase activity of the nanometer HA, it was easy to gather into bigger granules conglomeration, which enter cells through 'swallowing'.

In conclusion, our experiment provides information concerning the effect of nanometer hydroxyapatite on proliferation and ALP activity of cultured periodontal ligament cells, which may reflect a key contribution to periodontal reconstruction. During the process of periodontal regeneration, bone reconstruction plays an important role. The reconstruction of alveolar bone favors regeneration of a periodontal ligament which is attached to both new cementum and alveolar bone. ALP plays a key role in the formation and calcification of hard tissues, and its expression and enzyme activity are frequently used as markers of osteoblastic cells. The high expression of ALP activity in the nanometer HA group showed that nanometer HA had the ability to induce osteogenic differentiation of PDLC. On the other hand, the proliferation capability of PDLC also increased under the influence of nanometer HA, and the TEM investigation indicated the better biological compatibility and dissolvability of nanometer HA. In short, nanometer HA was likely to promote periodontal regeneration through the reconstruction of alveolar bone and the proliferation of PDLC and may thus be a suitable bone graft material for periodontal intrabony defects.

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