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Effects of EMPs on Human Dental Pulp Stem Cells *In vitro*

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The aim of this study was to isolate and characteristics human dental pulp stem cells (hDPSCs) derived from human third molar pulp. Another aim was to verify the expression with dentin sialoprotein (DSPP), dentin sialoprotein (DSP), dentin matrixprotein 1 (DMP-1) and vimentin. For characterisation, proliferation capacity, phenotypic properties, and differentiation characteristics were utilised. Stem cells isolated from hNDP were analysed by flow cytometry and immunocytochemistry. Cell line were directionally differentiated towards adipogenic, osteogenic chondrogenic, myogenic and neurogenic lineages. The expression of DSPP,DSP,DMP-1and vimentin, as analyzed by Immunocytochemistry , increased after EMPs incubating for 5d. So exhibit multilineage differentiation properties. Thus, both EMPs are important during hDPSCs proliferation. During cytodifferentiation, EMPs are essential for the complete differentiation of odontoblasts by up-regulating the expression of DSPP, DSP, DMP-1 and vimentin. Stimulation of these cells by EMPs significantly increases alkaline phosphatase(ALP) activity. ALP activity was expressed as OD 405 nm/mg of protein A increase was observed in ALP activity of hDPSCs during the culturing of EMPs. According to the differentiation and proliferation potential of HDPSC was shown. A specific function for EMPs up-regulat in differentiation of hDPSCs was elucidated.

Key words: Dental pulp stem cells, EMPs, DSPP, DSP , DMP-1, vimentin.

Human dental pulp stem cells (hDPSCs)derived from dental pulp have become increasingly popular to study. Detailed in vivo and in vitro studies were previously carried out to examine the proliferation and differentiation capacity of hDPSCs. Gronthos *et al*. (2000) identified a population of human dental pulp stem cells (DP-SCs) that can develop into odontoblasts, the cells that form the mineralised matrix of dentin. In other studies, researchers induced the differentiation of stem cells isolated from dental pulp (DPSCs) derived from deciduous teeth or wisdom teeth into various cell types (Nosrat *et al*. 2001; Gronthos *et al*.2002; Ikeda *et al*. 2006;Struys *et al*. 2007; Huang *et al*.2008). The dental pulp is a loose connective tissue containing various subpopulations of cells, including ûbroblasts and undifferentiated cells capable of transformation into cells capable of synthesis of mineralized tissue (Shi and Gronthos, 2003).Overall,experiments revealed that stem cells and/or precursor cells derived from dental pulp can differentiate into dentin-pro-ducer odontoblasts, osteoblasts, adipocytes, skeletal and/or smooth muscle cells, endothelial cells,neural cells and elastic cartilage cells both in vivo and in vitro (Gandia *et al*.2008; Gronthos *et al*. 2000, 2002;Huang *et al*. 2008;Jo *et al*.2007; Laino *et al*.2005; Miura *et al*. 2003;Otaki *et al*. 2007; Papaccio *et al*. 2006; Yu *et al*. 2007). Enamel matrix protein (EMPs; immature enamel extracts from porcine teeth used in periodontal regenerative therapies) have recently been shown

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to induce cementoblast differentiation and periodontal regeneration in vivo (Bosshardt *et al*. 2005) and are suggested to enhance cell proliferation, migration adhesion, and differentiation in vitro (Gestrelius *et al*. 1997; Rincon *et al*.2005). EMPs are essentially composed of amelogenins, which are enamel matrix proteins shown to have growth factor-like activity (Veis *et al*., 2000). In addition, EMPs increase alkaline phosphatase (ALP) activity and matrix mineralization in human periodontal ligament cells and osteoblasts and in rodent bone marrow stromal cells (Van der Pauw *et al*. 2000; Keila *et al*. 2004). Our results provide new information regarding the effect of EMPs on odontoblast differentiation and suggest the potential of using new growth factors. The aim of this study was to isolate and characteristics hDPSCs derived from human third molar pulp . Investigate the effect of EMPs on the expression of dentin sialoprotein (DSPP), dentin sialoprotein (DSP), dentin matrixprotein 1 (DMP-1)and vimentin.

MATERIALS AND METHODS

Isolation and culturing of hDPSCs

Human dental pulp cells derived from the dental pulp tissue obtained from a lower third molars extracted from a man(18 years old).The teeth were immersed in a physiological solution containing 100 IU/ml penicillin and 100ìg/ml streptomycin eliminate any contamination.Then under sterile conditions, use pliers to fracture the dental crown to make the dental pulp uncovered. The pulp tissue was digested in a solution of collagenase type I (Sigma-Aldrich, St. Louis MO) to generate single cell suspensions.The cells were cultured in DMEM/F12 containing 15% foetal bovine serum (FBS; Invitrogen/GIBCO, Grand Island, NY, USA) and 100 IU/ml penicilin-100ìg/ml streptomycin (Invitrogen/GIBCO). The cells from the teeth were seeded into two 25 cm2 plastic tissue culture flasks (BD Biosciences) and incubated at 37°C in a humidified atmosphere of 5% CO2 for 3 days. On the third day, non-adherent cells were removed. The adherent cells were continuously propagated until grown to 80% confluency. The fresh medium was added and replaced every 3 days over a 10–14 day period. Passaging of the cells was performed as described above.

Phenotype identification

Flow cytometry

To confirm that hNDP-SCs maintain their phenotypic characteristics after growth in culture,undifferentiated SCs were subjected to flow cytometry analysis. After each passage, stem cells were harvested and suspended in their own culture medium at a concentration of 1×10^6 cells/ml. Flow cytometry was performed and the data were analysed. Immunophenotyping of hNDP-SCs was performed with antibodies against the following human antigens: CD3, CD8, CD10, CD11b, CD13, CD14, CD15, CD19, CD33, CD34, CD44, CD45, CD71, CD73, CD90, CD117, CD146,CD166, and HLA-DR.

Cell proliferation and viability-MTT Test

The proliferation rate of hNDP-SCs was determined by MTT. MTT was analysed using the MTT Cell Growth Kit (Chemicon) on passage three cultures of hDPSCs. hDPSC suspensions were seeded at 12,500 cells/well in 6-well plates (for 1, 4, 7, and 11 days) and T25 flasks (for 14, 17,and 21 days) and were incubated in 5% CO2 at 37°C for 1, 4, 7, 14, 17, and 21 days. Next, the culture medium was replaced. Wells were washed twice with PBS. In the growth curve experiment, 10ìl MTT (0.5 mg/ml) was added to the culture for an incubation time of 4 h. Culture medium was discarded and replaced with 100ìl isopropanol/HCl. Absorbance at 570 nm was measured. For each group, experiments were repeated three times and measurements were completed in triplicate.

Cell differentiation in vitro

To test the effects of EMPs, hDPSCs were seeded into 24-well plates at a density of 10^4cells/well, cultured in basal medium with 15% FBS until confluence, and then maintained in basal or differentiation medium (basal medium with DMEM/F12ÿ15ÿPBS,) and agonist as follows: 200¼g/ml EMPs into hDPSCs, DSPP,DSP,DMP-1and vimentin investigated with immunohistochemistry stain in 3d05d07d09d011d as indicated.

Adipogenic adipogenic, osteogenic chondrogenic, myogenic and neurogenic lineages differentiation

Cells from passage three (3,000 cells/cm^2) were separately seeded onto coated type I collagen coverslips in 6-well plates to induce adipogenic , osteogenic, chondrogenic, myogenic and neurogenic lineages differentiation. The medium

of adipogenic and osteogenic were MEM (Invitrogen/GIBCO), and the medium of chondrogenic, myogenic and neurogenic were separately high-glucose DMEM, H-DMEM, L-DMEM . In adipogenic differentiation was supplemented with EMPs, 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methylxanthine (IBMX-Sigma-Aldrich), 10cþ6M dexamethasone, 10 g/ml insulin, 200¼M indomethacin, and 1% antibiotic/antimycotic. In osteogenic differentiation was supplemented with EMPs, 100 nM dexamethasone, 0.05¼M ascorbate-2-phosphate, 10mM²-glycerophosphate, 1% antibiotic/antimycotic and 10% FBS. In chondrogenic differentiation was supplemented with EMPs ,10 ng/ml transforming growth factor-1, 50ìg/ml ascorbate-2-phosphate, 0.1ìM dexamethasone, 100ìg/ml sodium pyruvate, 40ìg/ml proline, 50 mg/ml ITS premix, and 1% antibiotic/antimycotic. In myogenic differentiation was supplemented with EMPs, 5% horse serum and 10 mM 5-azacytidine. In neurogenic differentiation was supplemented with EMPs, 0.5 mM IBMX, 10 ng/ml BDNF, EGF, and (â-FGF).The cells were incubated for 4 weeks and the medium was replaced twice a week. In order to assess their differentiation,cells staining was utilized.

Immunocytochemistry analysis

Cells were incubated overnight at 4°C,5% CO2 at 37°C with the primary antibodies of DSPP,DSP,DMP-1and vimentin . The following day, cells were incubated with biotinylated secondary antibodies for 15 min at room temperature. Incubations were followed by streptavidin peroxidase treatment for 15 min at room temperature and signals were detected with the AEC kit (Zymed Laboratories, UK). The cells were counter-stained with haematoxylin (Santa Cruz Biotechnology) and were examined under a light/fluorescent microscope (Leica DMI 4000B, Wetzlar, Germany).

Quantification of ALP activity

At 3,5,7,9, and 11 days in differentiation medium, hDPSCs layers were rinsed with PBS, scraped off into 50 mM TRIS-HCl pH 7.4, containing 0.1% Triton X-100 (Sigma), and sonicated for 1 min. Then the cells were incubated at room temperature with p-Nitrophenyl Phosphate for 30 min. The optical density of the product, para-nitrophenol, was determined by a VersaMax Microplate Reader (Molecular Devices) at 405 nm. ALP activity was expressed as OD 405 nm/mg of protein (Wu *et al*. 2007).

Statistical analysis

Results were expressed as mean ±SD and all statistical analyses were performed using SPSS 12.0. All experiments were repeated three times. Results were analysed using one-way ANOVA and the paired t test. Differences between the experimental and control groups were regarded as statistically significant when $p < 0.05$.

RESULTS

Cultures of hDPSCs hDPSCs displayed a fibroblast-like, spindle-shaped morphology during their early days of incubation. These cells began to proliferate after 3–4 days of incubation and gradually grew to form small colonies .These primary cells reached 70–80 % confluency after 12–15 days of incubation during their early passages. During later passages, the morphology of majority of cells exhibited large, flattened or fibroblast-like.

Flow cytometry-identification of hNDPSCs

Defined markers exist that specifically and uniquely identify MSCs. We utilised some markers to define our cultured cells. Our data indicated that hNDP-SCs expressed CD13, CD44, CD73, CD90, CD146, and CD166,but not CD3, CD8, CD10, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD71, CD117, and HLA-DR. These findings are consistent with their undifferentiated state.

Table 1. ALP activity of hDPSCs P<0.05

group	ALP activity (A410)				
	3d	5d	7d	9d	11d
contral	0.256±0.003	0.268±0.015	0.285±0. 008	0.297±0.019	0.306±0.024
100μg/ml	0.342±0.012	0.387±0.011	0.411±0. 007	0.458±0.016	0.445±0.013
200μg/ml	0.468±0.006	0.502±0.008	0.531±0. 013	0.578±0.007	0.565±0.012

Growth characteristics of hNDP-SCs

Under the culture conditions used in this study, hNDP-SCs were consistently grown for more than 25 passages without losing their proliferative ability. The data from the MTT assay showed that the mean percentage of formazan absorbance values (OD values) increased .

Immunocytochemistry-identification of hNDPSCs

Immunocytochemical staining of hNDPSCs was depicted in this study. Vimentin is a popular cytoplasmic marker used to denote hNDPSCs and it was expressed throughout the cell passages in this study. Under the standard culture conditions, these cells expressed other MSC markers such as CD44, CD105/endoglin and fibronectin and their morphological characteristics remained unchanged. hNDP-SCs did not express other surface markers such as CD31, CD34, CD45 and CD71. In addition, hNDP-SCs that were not stimulated towards differentiation, expressed some myogenic markers such as desmin, myogenin, and myosin IIa, and chondrogenic markers including type II collagen. The hNDP-SCs used in this study were positive for GFAP, an intermediate filament protein specific for astroglial cells. In addition, they expressed neural progenitor markers such as nestin, vimentin and neuronal markers such as c-fos, beta III tubulin and MAP2a,b.

Differentiation potential of hNDP-SCs

We use histochemical and immunohistochemical methods to determine the potential of hNDPSCs to differentiate to adipogenic, osteogenic, chondrogenic, myogenic and neurogenic states. hNDPSCs at passages 3–5 were used in the adipogenic differentiation experiments, At the start of the fifth week, the lipid droplets enlarged and invaded the entire cytoplasm like adipocyte-differentiated hNDPSCs. Detected bone-specific markers were applied to cells grown in osteogenic differentiation. Alizarin red positive nodular aggregates were present at day 28. And in later positive alizarin red aggregates were larger and stained intensively, indicating that calcium deposition occurred. After 3 weeks, the cell pellets that were induced to differentiate into chondrocytes stained positively with alcian blue. Hypertrophic cells were readily identifiable. Cells were round and resembled hyalin chondrocytes, forming cartilaginous lacunae . hNDPSCs were treated with 5-azacytidine to acquire a myocyte phenotype in culture. The myogenic cells were strongly positive for all myogenic lineage markers. During Neurogenic differentiation, we realised the morphological of cells changes to appear neuron-glial-like,the immunohistochemical staining intensities for all neuronal markers increased.

Expression of DSPP,DSP,DMP-1and vimentin

The immunohistochemical method was used to determine the expression of DSPP, DSP, DMP-1and vimentin. We use passage 5 from hNDPSCs and treat them with 200ìg/ml EMPs to acquire positive expression. DSPP, DSP were

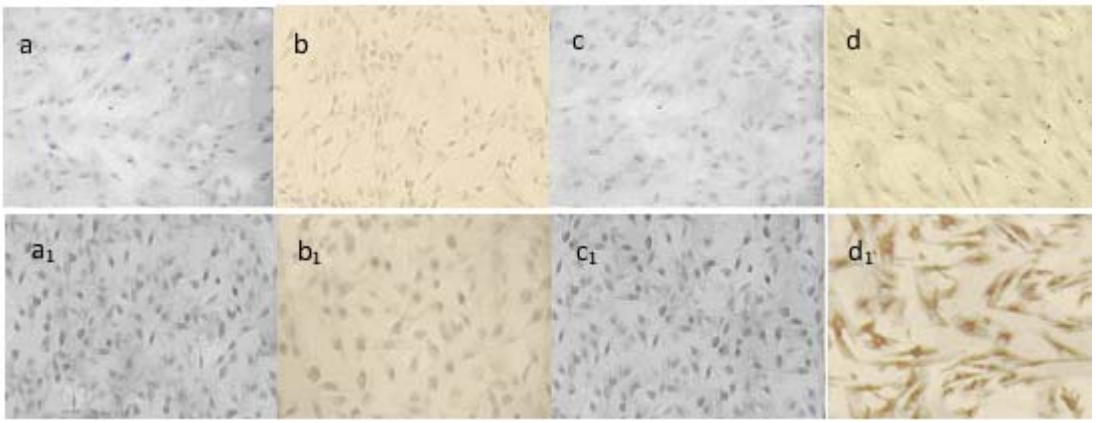

Fig. 1. Immunophenotype staining of cultured hNDP-SCs. Studies based on immunoperoxidase reactivity were performed on third passage cultures of hNDP-SCs. Representative staining patterns are shown for: DSP (a), DSPP (b), DMP-1 (c)and vimentin (d),and DSP (a_1), DSPP (b_1), DMP-1 (c_1),and vimentin (d1)with EMPs incubating of hNDP-SCs. Control a few number of cells (less than 20 %) stained positive for DSP (a), DSPP(b), and no stained positive for DMP-1 (c)and vimentin (d). After 200ìg/ml EMPs into hDPSCs 5days stained positive for DSP (a_1), DSPP (b_1), DMP-1 (c_1)and vimentin (d_1)

sparse expression ,DMP-1and vimentin were negative expression in control group, and after 5 days incubating with 200ìg/ml EMPs in the medium positive expression in experimental group. (Fig. 1)

Alkaline phosphatase activity

As a marker for odontoblast differentiation, ALP levels were measured. ALP activity increased following a 3-day incubation in odontogenic differentiation medium when compared with cells exposed to regular growth medium ALP activity increased until day 9 and then decreased. A twofold increase was observed in ALP activity of undifferentiated hDPSCs during the first 9 days of culturing. (Tab.1)

DISCUSSION

Firstly Gronthos and colleagues, in 2000, isolated stem cells from human dental pulp, calling them DPSCs (dental pulp stem cells). These cells exhibited differentiation potential into odontoblasts, the cells that form the mineralised matrix of dentin.

We studied the potential of hDPSCs to differentiate into odontoblasts. Several authors have suggested the use of EMPs for periodontal regeneration. In vitro, EMPs enhance the expression of calcified-matrix markers in mouse periodontal or bone marrow mesenchymal cells and in human adult periodontal ligament cells (Hakki *et al*.2001; Keila *et al* 2004). However, the effects of EMPs on hDPSCs differentiation are unknown. We have demonstrated that induce by EMPs, hNDPSCs expressed osteogenic, chondrogenic, adipogenic, myogenic and neurogenic markers under basal conditions, and have the capacity to differentiate into mature adipocytes, myoblasts, neuro-glial cells, chondroblasts and osteoblasts in vitro under permissive differentiation conditions. Most importantly, we show here that EMPs up-regulate the expression of DSPP, DSP , DMP, 1and vimentin by hDPSCs. This result may indicate their intrinsic tendency of differentiation towards odontoblasts.

ALP serves as a useful marker for the transition from the proliferative period to matrix maturation in odontogenesis. In our study, a time-dependent effect of osteogenic medium on ALP activity was observed. ALP activity increased in the control cells and in the cells induced for odontogenic differentiation after 3 days of culturing. ALP activity increased approximately twofold after 9 days of culturing and continued to increase. After 14 days of culturing, the activity stopped rising and started to decrease. Our results provide evidence that ALP are increased by the addition of EMPs to the culture medium.These results are supported by the findings of previous studies (Myginda *et al*. 2007; Bjerre *et al*. 2008). However, clonally selected cells still need to be tested to confirm this result.

This study reports, for the first time, that EMPs play a pivotal role in the differentiation of hDPSCs toward the odontoblast phenotype, at the initiation of dentin morphogenesis.

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