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Human dental follicle cells express embryonic, mesenchymal and neural stem cells markers

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ABSTRACT

Objective: This study was conducted to identify and characterize dental follicle stem cells (DFSCs) by analyzing expression of embryonic, mesenchymal and neural stem cells surface markers. Design Dental follicle cells (DFCs) were evaluated by immunocytochemistry using embryonic stem cells markers (OCT4 and SOX2), mesenchmal stem cells (MSCs) markers (Notch1, active Notch1, STRO, CD44, HLA-ABC, CD90), neural stem cells markers (Nestin and β-III-tubulin), neural crest stem cells (NCSCs) markers (p75 and HNK1) and a glial cells marker (GFAP). RT-PCR was performed to identify the expression of OCT4 and NANOG in DFCs and dental follicle tissue.
Results: Immunocytochemistry and RT-PCR analysis revealed that a significant proportion of the DFCs evaluated expressed human embryonic stem cells marker OCT4 (75%) whereas NANOG was weakly expressed. A considerable amount of MSCs (90%) expressed Notch1, STRO, CD44 and HLA-ABC. However, they were weakly positive for CD90. Moreover, it was possible to demonstrate that dental follicle contains a significant proportion of neural stem/progenitors cells, expressing β-III-tubulin (90%) and nestin (70%). Interestingly, immunocytochemistry showed DFCs positive for p75 (50%), HNK1 (<10%) and a small proportion (<20%) of GFAP-positive cells. This is the first study reporting the presence of NCSCs and glial-like cells in the dental follicle.
Conclusions: The results of the present study suggest the occurrence of heterogeneous populations of stem cells, particularly neural stem/progenitor cells, in the dental follicle, Therefore, the human dental follicle might be a promising source of adult stem cells for regenerative purposes.

1. Introduction

Mesenchymal stem cells were first identified in aspirates of adult bone marrow. Initially, clonogenic clusters of adherent fibroblastic colony-forming units with the potential to undergo extensive proliferation in vitro and to differentiate into different mesenchymal cell lineages were developed (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). Since then, bone marrow had been the most utilized source of MSCs. Nevertheless, there was a need to isolate MSCs from accessible tissues with minimal surgical trauma.

The dental follicle is a loose ectomesenchymally derived connective tissue surrounding the enamel organ and dental papilla of the developing tooth germ. It coordinates the tooth eruption and harbours progenitor cells for the periodontium (Ten Cate, 1997). Dental follicle represents an appealing source of stem/progenitor cells owing to the fact that it is an expendable tissue that can be removed with minimal morbidity (Ikeda et al., 2006). Through tissue engineering, those stem cells could be exploited to generate more cellular material for tissue repair than could be generated in situ, during the lifetime of an organism (Gronthos, Mankani, Brahim, Robey, & Shi, 2000). This feature confers great potential for the application of such tissue in cell therapy (Ikeda et al., 2006).

Dental follicle cells have multipotential mesenchymal precursor cell properties after differentiating toward multiple mesenchymal-derived cell types, such as cementoblasts, chondrocytes, adipocytes (Kémoun et al., 2007) and osteoblasts (Morsczeck et al.,

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2005). Recent studies have demonstrated that dental follicle cells can differentiate into neural-like cells, when cultured in a neural inductor medium (Völlner, Ernst, Driemel, & Morsczeck, 2009). Nonetheless, the immunophenotype as well as the capacity of neural differentiation of DFSCs has not been extensively explored. Hence, the aim of this study is to characterize DFSCs by analyzing the expression of various cell surface markers used to identify putative embryonic, mesenchymal and neural stem cells.

2. Materials and methods

2.1. Individuals and sample

Six individuals aged between 14 and 16 years were selected to volunteer in the study, according to the following inclusion criteria: a- healthy subjects, with good oral health condition, referred for third molar extraction due to orthodontic reasons and b- presence of impacted third molars with roots in the initial calcification phase, between stages six and seven of tooth development, observed on panoramic radiographs (Nolla, 1960).

The sample included 16 fresh human dental follicles. The region of interest in the dental follicle comprised the part that surrounded the developing roots. Each dental follicle was carefully separated from the roots with aid of a sterile forceps and a scalpel and then washed in phosphate buffered saline (PBS) to remove blood. The tissue remained immersed in solution of chlorhexidine gluconate 0.12% during one minute for bacterial decontamination, and then was washed again in PBS. This study was approved by the Ethics Committee on Human Research of the University Hospital Clementino Fraga Filho of Federal University of Rio de Janeiro (protocol number: 06225113.6.0000.5257) and was conducted according to ethical principles for research involving human subjects of the World Medical Association (Declaration of Helsinki).

2.2. Histological analysis

The follicular tissue was fixed in 4% solution of paraformaldehyde/0,1 M PBS at 4 °C for 48 h. Then, the tissue was dehydrated in ascending series of ethanol and embedded in paraffin. Serial sections of 5 μm were cut in different planes and stained with hematoxylin-eosin (HE) and Gomori trichrome for tissue characterization. Staining with periodic acid-Schiff (PAS) was performed to identify glycoproteins in the extracellular matrix.

2.3. Cell culture experiments

The follicular tissue was cut into small fragments with the aid of sterile scissors and forceps. Then, tissue fragments were enzymatically digested in a solution containing 0.1 U/ml collagenase type II (Sigma-Aldrich) at 37 °C for one hour. Inactivation of collagenase was performed with 1 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Life Technologies). This solution was centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml DMEM/F12 with 10% FBS. Minced and digested tissues of dental follicle explants were seeded into T25 flasks in DMEM/F12, 20% FBS, 100 μm L-ascorbic acid phosphate (Sigma-Aldrich), antibiotics/antimycotic (a/a; 100 U/ml penicillin, 0,1 mg/ml streptomycin; Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies) at 37 °C in 5% CO_2 in a humidified atmosphere. After single cells have attached to the plastic surface, non-adherent cells were removed by changing the medium every 2 days. Plastic adherent cells were propagated until they reached approximately 80% of confluence. During this period, dental follicle cells were examined by phase-contrast microscopy and cells from passages 1 to 5 were used for all experiments. Early passages were used because seems that asymmetric dividing progenitor cells ratify after passaging (Morsczeck et al., 2005; Sherley, 2002).

2.4. Immunocytochemistry experiments

Cells were fixed with 4% paraformaldehyde pH 7,4 for 30 min at room temperature and permeabilized with 0.2% Triton X-100 (Reagan) in PBS for 5 min. For blocking, cells were treated with 5% bovine serum albumin (BSA, Gibco) in PBS for 1 h. Cells were incubated with primary antibodies (Table 1) overnight at 4 °C and then with specific secondary antibodies conjugated to Alexa Fluor 488, 546 or 647 (Invitrogen) for 2 h. Nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0,25 μg/μl (Sigma-Alderich) for 5 min. For negative controls, primary antibodies were omitted and only secondary antibodies were used. Epifluorescence observation and photodocumentation were accomplished using Leica confocal microscope. The proportion of stained cells relative to unstained was expressed in percentage. Fluorescence intensity of markers was classified as low, moderate or high according to Leica

Table 1
Primary antibodies used in immunocytochemistry experiments.

Primary antibodies	Antigen	Classification	Species	Sources	Dilution
Anti-Active Notch1	Active Notch1 receptor	Polyclonal	rabbit	Abcam	1:500
Anti-CD44	CD44 (HCAM-1)	Monoclonal	mouse	BD-Pharmingen	1:100
Anti-CD90	CD90 (Thy-1)	Monoclonal	mouse	BD-Pharmingen	1:200
Anti-Fibronectin	Fibronectin	Polyclonal	rabbit	Sigma	1:200
Anti-GFAP	Glial fibrillary acidic protein	Polyclonal	rabbit	DAKO	1:500
Anti-HLA-ABC	Human leukocyte antigen-ABC	Monoclonal	mouse	BD-Pharmingen	1:200
Anti-HNK1	Human natural killer-1	Monoclonal	hybridoma	Laboratory	It is not fixed
Anti-Nestin	Nestin	Monoclonal	mouse	Chemicon	1:100
Anti-Nocht1	Notch1 receptor	Monoclonal	mouse	Santa Cruz	1:50
Anti-OCT4	Octamer-binding transcription factor 4	Monoclonal	mouse	Millipore	1:100
Anti-p75	Low-affinity nerve growth factor receptor or p75 neurotrophin receptor	Monoclonal	mouse	Millipore	1:500
Anti-Phalloidin	Phalloidin	Monoclonal	mouse	Abcam	1:200
Anti-SOX2	Sex determining region Y (SRY)	Polyclonal	rabbit	Millipore	1:100
Anti-STRO1	STRO1 receptor	Monoclonal	mouse	Invitrogen	1:200
Anti-Vimentin	Vimentin	Monoclonal	mouse	DAKO	1:200
Anti-α-SMA	α-smooth muscle actin	Monoclonal	mouse	DAKO	1:100
Anti-β-III-tubulin	β-III-tubulin	Monoclonal	mouse	Promega	1:1000