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Evaluation of immunophenotyping, proliferation and osteogenic differentiation potential of SSEA-4 positive stem cells derived from pulp of deciduous teeth

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ABSTRACT

Objectives: Despite the increased interest in stem cells isolated from remnant pulp of deciduous teeth, no specific marker has been yet established for them. The present study aimed to investigate whether SSEA-4 (stage-specific embryonic antigen) would be a suitable marker to isolate stem cells from Human Exfoliated Deciduous teeth (SHEDs) in order to increase its differentiation potential toward osseous tissue.
Design: The SHEDs were isolated and the expression patterns of mesenchymal, hematopoietic and embryonic stem cell markers were assessed. The cells were then divided into two groups of SSEA-4(+) and unsorted SHEDs and the cell proliferation rate and population-doubling-time (PDT) were calculated. Subsequently, the differentiation potentials were examined through alizarin-red staining and Quantitative real time-PCR (qRT-PCR).
Results: Isolated cells were spindle-shaped with a high expression of mesenchymal stem cell markers and weak expression of hematopoietic markers. The mean expression of Oct-4 was 68.77% ± 1.28. Despite similar proliferation rates between SSEA-4(+) and unsorted SHEDs, because of differences in the shape of the growth curves, PDT was lower in unsorted SHEDs ($P = 0.2 \times 10^{-4}$). Alizarin-red staining showed similar calcium deposition in both groups. Upon differentiation, the expression of osteocalcin was higher in unsorted SHEDs ($P = 0.043$), while, the expression of alkaline phosphatase was lower ($P < 0.001$). The parathyroid hormone receptor (PTHR) expression was not significantly different ($P = 0.0625$).
Conclusions: The results of the present study revealed that SHEDs have high differentiation potentials even in the unsorted cells. Although the SSEA-4-positive SHEDs showed slightly better osteogenic potential, the differences were not abundant to link SSEA-4 expression with superior differentiation potency.

1. Introduction

One of the setbacks in dental treatments is repair of damaged alveolar bone. Although the use of stem cells in regenerative dentistry has been promising; a well-defined therapeutic protocols have not yet been achieved. Stem cells from Human Exfoliated Deciduous teeth (SHED) have been identified as a promising source of mesenchymal stem cells (MSC) since it was first reported by Gronthos et al. (2002). SHEDs are clonogenic and highly proliferative cells capable of self-renewal and multi-lineage differentiation (Gronthos et al., 2002). In addition, these stem cells can be also isolated in a non-invasive manner compared to the other adult tissue sources (Tirino, Paino, & De Rosa, 2012). SHEDs have been found to grow more rapidly compared to adult dental pulp stem cells (DPSCs) and bone marrow stem cells BMSCs. This might be because they are less mature than other stem cells found in the body (Tsagias et al., 2014).

In order to isolate SHEDs, most studies have employed enzyme digestion technique since this method leads to stem cells with higher mesenchymal surface marker and differentiation potentials (Bakopoulou et al., 2011). Identification and isolation of certain stem cells with a high regenerative capacity could lead to a more reliable and consistent tissue formation and cell surface markers can be employed to obtain cells with specific surface characteristics (Niemeyer, Pestka, Salzmann, Sudkamp, & Schmal, 2012).

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SSEA-4 (stage-specific embryonic antigen) is a glycolipid carbohydrate epitope and has been employed as a marker for recognizing primitive human embryonic carcinoma cells and embryonic stem cells (Kannagi et al., 1983). Studies have also shown that the expression of SSEA-4 was down-regulated following differentiation of human ES and EC cells (Draper, Pigott, Thomson, & Andrews, 2002; Gang, Bosnakovski, Figueiredo, Visser, & Perlingeiro, 2007). Consequently, there is a general assumption that a direct relation exists between expression of embryonic stem cell markers and factors defining the "stemness". Gang et al. (2007) found a correlation between the SSEA-4 and stemness of BMSCs. They have also demonstrated that SSEA-4 identifies adult mesenchymal stem cell population in human bone marrow aspirate.

Despite the increased interest in stem cells isolated from remnant pulp of deciduous teeth, no specific marker has been yet established for them. They are generally defined on a functional basis including culture adherence, and their ability to self-renew and to differentiate into at least one mature cell type. Also, based on our search in the literature, the stemness quality of the SHEDs isolated based on the SSEA-4 marker expression have not been compared to the unsorted cells. Only two investigations (Kawanabe et al., 2012, 2015) isolated the SHEDs based on the SSEA-4 marker expression, however, the qualities of isolated cells have not been compared to the unsorted cells in both studies.

Therefore, the aim of the present study was to investigate whether isolating stem cells from deciduous pulp tissue based on SSEA-4 marker would result in a more primitive stem cell population with increased differentiation potential toward osseous tissue compared to the unsorted cells.

2. Materials and methods

Information about materials used in this study is as follows: fetal bovine serum (FBS), Fungizone, dispase, L-glutamine and non-essential amino acids (NEAA) were supplied by Gibco BioCult (Paisley, Scotland, UK). Alpha Modification Minimum Essential Medium Eagle (α-MEM), Dulbecco's modified Eagle's medium-F12 (DMEM-F12), DMEM-low glucose (LG), DMEM-high glucose (HG), penicillin, streptomycin, collagenase I, trypsin, EDTA, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dexamethasone (DEX), β-glycerophosphate (β-GP), ascorbic acid, and alizarin red were purchased from Sigma-Aldrich (St. Louis, MO, USA). Super ScriptTM II Reverse Transcriptase DNAse obtained from Life Technologies (CA, USA). N6 Random-Hexamer, dNTP Mix, First Strand buffer, and RiboLockTM RNase inhibitor were purchased from, Fermentas Inc(MD, USA) and RNEasy mini kit was acquired from Qiagen Inc, (CA, USA). SYBR Premix EX TaqTM was provided by Takara Bio Inc, Japan. Antibodies used in this study were purchased from either BD Biosciences (San Jose, CA) or Sigma-Aldrich (St. Louis, MO, USA).

2.1. Sample preparation

The samples were collected under a protocol that was approved by the Medical Ethics Committee of the National University (ethical approval ID. IR.TUMS.REC.1394.1945) and informed consent was obtained from the parents of all donors. The exfoliating deciduous teeth were extracted from 5 healthy children aged from 9 to 12-years. The extraction of the teeth was in their treatment plan because of space management, malposition, and etc. People involved in the diagnosis and treatment planning had no participation in the present study.

2.2. Isolation and culture of SHEDs

After the extraction, stem cells were isolated as previously reported techniques (Aghajani et al., 2016). Briefly, dental pulp was removed through the root resorption site and single cell suspensions were prepared by immersing in a digestive solution of collagenase I (4 mg/ml)/dispase (3 mg/ml) for 1 h at 37 °C followed by filtering the solution with 70-μm Falcon strainers. Then, the cells were cultured in DMEM-F12 with 15% FBS and incubated at 37 °C and 5% CO_2.

2.3. Flow cytometric immnunophenotyping

Cells at passage 2–3 were used for immunophenotyping. The following antibodies were assessed according to the manufacturers' instructions; hematopoietic surface markers of CD34, CD38, CD45; mesenchymal markers of CD29, CD44, CD73, CD133, CD146, CD105, STRO-1, and embryonic markers of SSEA-4 and Oct-4. Cells were also labeled with isotype control antibodies of phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated.

In brief, aliquots of single cell suspension (2×10^5 cells/100 μl) were prepared. Each aliquot assigned to one of the antibodies and incubated with the specific FITC- or PE-labeled antibodies for 45 min at 4 °C. The expression of Oct-4 was determined through intracellular flow cytometry staining in which detached cells were fixed with 4% formalin for 15 min at room temperature before they were washed twice with a permeabilization buffer. The cells were then incubated with anti-human Oct-4 (1:1000) for 40 min at 4 °C and subsequently in FITC-conjugated goat anti-rabbit Ig (diluted 1:150 in permeabilization buffer) for another 40 min at 4 °C.

In order to wash away any unbound antibodies, the cells were washed twice by PBS-FBS 2% and then analyzed using flow cytometry (Partec, Germany). The obtained data were analyzed using FlowJo (Tree Star, Ashland, OR, USA).

2.4. Fluorescent-activated cell sorting (FACS)

In order to select SSEA-4(+) cells, single cell suspension containing at least 3×10^6 cells were incubated with the SSEA-4(PE-conjugated) antibodies for 40 min at 4 °C and then washed twice with PBS-FBS 2%. With regard to the isotope control of PE-conjugated cells, SSEA-4(+) cells were collected using BD FACSAria II (BD Biosciences, San Jose, CA, USA). PE fluorescence was excited with a 488 nm laser and detected with a 585/42 nm filter.

2.5. Cell proliferation and population doubling time

The cell proliferation rate of sorted and unsorted SHEDs (S-SHEDs and U-SHEDs, respectively) was assessed using the MTT assay. In brief, 10^4 cells were seeded and cultivated for 1, 4, 7, and 10 days. At the end of each incubation period, the culture medium was discarded and 500 μl phenol red free media and 50 μl of 5 mg/ml MTT was added to each well. The cells were incubated in the dark for 4 h at 37 °C in 5% CO_2. Then, the insoluble formazan was dissolved with 200 μl DMSO at room temperature. The absorbance was measured at a wavelength of 570 nm by a microplate reader (Labsystem Multiskan, Finland).

The population doubling time (PDT) was determined by means of online application (Ruth, 2006) as explained in our previous study (Aghajani et al., 2016). The number of cells was calculated based on the cells' growth curve determined by MTT test.

2.6. Osteogenic differentiation

S-SHEDs and U-SHEDs were plated in 6-well plates at a density of 5×10^4 cells per well. When cells reached 75% confluency, the culture media was replaced with osteogenic medium consisting of DMEM-HG supplemented with 10% FBS, 0.1 mM DEX, 10 mM β-GP, and 50 mM ascorbic acid. The cultures were sustained for 3 weeks and the media was changed 2–3 times a week.

2.7. Alizarin red staining

After three weeks of differentiation, cultured cells were fixed by