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Assessment of human periodontal ligament stem cell surface molecules and wisdom tooth follicle stem cell surface molecules

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ABSTRACT

Objectives: Stem cells are the best cells that can be used for periodontal tissue regeneration in the treatment of periodontal disease. The aim of the present study was to compare the features of dental follicle stem cells (DFSCs) and periodontal ligament stem cells (PDLSCs).

Materials and Methods: In this study, five samples from DF and five samples from PDL were collected from patients. Cells were subsequently expanded by three passages. Cells were evaluated then by inverted microscope and flow cytometry. (DFSCs) and (PDLSCs) were stained with markers (CD90, CD166, CD105, CD44, CD73, CD13, CD45, CD34, CD38, and CD31) and examined for as well as for osteogenic and adipogenic differentiation.

Results: The DFSCs and PDLSCs expressed MSCs markers, as shown by flow cytometry. The cells were negative for CD45, CD34, CD38, CD31 markers but were positive for CD90, CD166, CD105, CD44, CD73, and CD13 markers. Cell attached to the flask macroscopically, and spindle cells attached to the inverted microscope. DFSCs and PDLSCs also differentiated into osteoblasts and adipocytes in osteogenic and adipogenic media.

Conclusion: This study opens the way for further research of human dental follicle and periodontal cells. The present study found that stem cells derived from the PDL and DF express CD90, CD166, CD105, CD44, CD73, and CD13 markers, similar to mesenchymal stem cells. PDL and DF cells differentiated into osteoblasts and adipocytes in osteogenic and adipogenic media.

Key words: Antigens, Mesenchymal stromal cells, Periodontal ligament.

Introduction

Periodontitis is a chronic inflammatory disease that causes destruction of tooth-supporting tissues, eading to tooth loss. Although several approaches have been used for periodontal regeneration, complete periodontal regeneration has not been achieved. Tissue engineering, using a combination of cells and scaffolds, is considered to be a lasting way to achieve complete regeneration [1-3]. Tissue engineering procedures have been developed for many tissues and organs, including bones, the heart, the liver, and the kidneys. By using tissue re-

generation, damaged periodontal tissue can also be regenerated by using stem cells, growth factors, and an extracellular matrix scaffold [4].

A stem cell (SC) is defined as a cell that can continuously produce unaltered daughters, and also has the ability to generate cells with different and more restricted properties [5-7]. Stem cells can be divided into two groups: embryonic and adult. Pluripotent stem cells can be isolated from the embryonic inner-cell mass [5-7]. Embryonic stem cells are pluripotent and able to diff ferentiation into almost any type of mature cell. However, ethical concerns regarding use of human embryos and the potential danger of teratoma formation have attributed to the limited application of these cells. So, more attention has focused on stem cells derived from adult tissues. Adult stem cells have a more confined differentiation potential compared with embryonic stem cells; however, adult stem cells are capable of exerting the basic functions of stem cells, such as self-renewal, the generation of large numbers of offspring, and differentiation into several mature cell types [8].

Mesenchymal stem cells (MSCs) derived from the bone marrow. MSCs express several markers: CD90 (Thy-1 (Thymocyte differentiation antigen 1)), CD13 (zinc metalloproteinaseis), CD73 (5'-ribonucleotide phosphohydrolase), CD105 (Endoglin) are markers for MSCs. Low level of these markers show MSCs: CD34 (glycoprotein), CD45 (Leukocyte common antigen), CD31 (clearing aging neutrophils). CD38 (cyclic ADP ribose hydrolase) are on lymphocytes and NK cell and plays a role in cell adhesion and intracellular calcium ion regulation. CD44 which is not present on the surface of dendritic cells and platelets plays role in cell-cell interaction and cell adhesion and migration. CD166 (a membrane glycoprotein) is a immunoglobulin. [9-12] MSC-like populations from other tissues are now discovered. The periodontal ligament (PDL) not only has a significant role in supporting teeth but also contributes to tooth nutrition, homoeostasis, and repair of damaged tissues. [13, 14] PDL contains heterogeneous progenitors, which can be activated to self-renew and regenerate periodontal tissues, such as the cementum and alveolar bone. [5, 15, 16]The dental follicle is an ectomesenchymal tissue surrounding the developing tooth germ. This tissue contains stem cells and lineage-committed progenitor cells for cementoblasts, periodontal ligament cells, and osteoblasts [17].

The aim of the present study was to compare the features of dental follicle stem cells (DFSCs) and periodontal ligament stem cells (PDLSCs).

Materials and Methods

Patient selection

Patients were selected from those referred to the maxillofacial and periodontal clinic of Tehran University of Medical Sciences, International campus. The present study performed according to the guidelines of the Helsinki Declaration of 1975 (revised, 2000). The research protocol was approved by the Ethics Committee of the Dental Research Center of Tehran University of Medical Sciences (IR.TUMS.REC.1395.2818). Informed consent was obtained from all participants, all of whom signed a form that was previously reviewed and approved by the patients. Fourteen Iranian women patients from 15–23 years old were chosen for the study. Five of them underwent surgery for impacted wisdom teeth, and the others underwent periodontal surgery. Patients completed personal and familial medical and dental history questionnaires; exclusion criteria were a history of smoking or alcohol use, or using any medication. Dental follicle and periodontal tissues were gained from the surgically excisional tissues routinely separated from dental roots. These tissues were transferred in DMEM (Dulbecco's modified Eagles medium) solution within 2 hours to the Iranian Tissue Bank Research & Preparation Center of the Farabi Hospital of Tehran University of Medical Science.

Isolation and culture of periodontal stem cells in the lab Samples were cut into several sections and were washed three times with a phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA) solution, then centrifuged for 10 min at 1500 rpm and cut into fine pieces. These pieces were digested with 0.1% collagenase IV (Nitta Gelatin, Osaka, Japan) for 1 h, centrifuged for 10 min at 1500 rpm, and were shaken vigorously at 37° C for 15 min. The resultant cell suspension was filtered through a 70 μm nylon mesh to remove tissue debris. Subsequently, collagenase was removed through dilution with PBS solution, and the cells were centrifuged twice for 5 min at 1500 rpm. Unfortunately, four specimens of PDLSCs failed to proliferate due to fungus contamination, so the Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) medium was supplemented by amphotericine B.

A 1 10^3 × matrix of cells were plated into a 25 cm^2 flask containing DMEM (Sigma) supplemented with low-glucose DMEM (Sigma, USA), 10% PBS, glutamine-L, 100 U penicillin, 100 mg/ml streptomycin, 5 mg/ml amphotrypcine B, and 0.05% ethylene diamine tetraacetic acid (EDTA) for passage.

During the next 72 hours, a microscopic assay was performed every three hours. The culture medium was changed after 24 hours and thereafter every four days. When 4/5 of the bottom of the flask was covered with cells, the cell passage was performed, using 0.25% trypcine containing 1 mM EDTA (Gibco BRL), and passaged at 80% confluency. The cells incubated at 37˚ C in an atmosphere of 5% CO_2.[18-20]

Identification of stem cells by flow cytometric analysis

Fluorescence-activated cell sorter (FACS) was performed using CELL QUEST software to analyze the surface markers of isolated human DF (DFSCs) and human PLSCs (hPLSCs). First, the cells were treated using 0.25% EDTA-trypcine and counted using neobar lam, then centrifuged. A collection of 10^5–10^6 cells were divided into two units containing 3% PBS, which made the culture medium suitable for adding a monoclonal antibody and the cell matrix was placed at room temperature for 30 min. Again, the cells were centrifuged at 1000 rpm for 6 min, and PBS was added to the solution. The cell mixture was passed through a nylon mesh, and PBS was added. Then the cells were conjugated with anti-CD166, anti-CD45, anti-CD34, anti-CD90, anti-CD13 Fluoresce iniso thio cyanate (FITC) and anti-CD105, anti-CD44, anti-CD73, anti-CD38, and anti-CD31 phyco erythrin (PE). The resultant mixture was placed at 4˚ C in the dark for 45 min and, after washing, was placed in the flow cytometry device (BD FACSCALIBUR™) [21].

Differentiation to osteoblasts

A sample of third passage PDLSCs and a sample of DFSCs were immersed in a solution of DMEM+10% FBS+dexamethasone (10nM)+β-glycerophosphate (10mM)+ascorbic acid 2-phosphate (50mg/mL) to differentiate into osteoblasts. The medium was replaced every two to three days.

After 14 days, the cultures were stained using the von Kossa staining. During the staining process, the samples were washed with PBS and fixed in 4% paraformaldehyde in PBS and were then washed with distilled water. After 10 minutes at room temperature, they were stained and washed again with distilled water and PBS at a pH of 7.2.

Differentiation to adipocytes

A samples of third passage PDLSCs and a sample of DFSCs were immersed in a solution of DMEM+10% FBS+dexamethasone (10nM)+indomethacin (50mg/mL) to differentiate into adipocytes. The medium was replaced every two to four days.

During the staining, the samples were washed with PBS and fixed in 4% paraformaldehyde in PBS followed by a rinse with distilled water. After three weeks, they were stained with 0.5% Oil Red O. [30]

Statistical Aanalysis

For statistical analysis the version of IBM® SPSS® 22 were used. Data was normally distributed. The parameters were compared between two groups using Paired sample t-test.

Results

Microscopic evaluation

During microscopic observation, samples from the isolated and cultivated PDLSCs and DFSCs showed polyhedral elongated cells with an oval nucleus in the center of the cells. These cells were attached to the flask, which were grown from the original stem cell's features (Figure 1 & 2).

Flow cytometric assessment (FACS)

Human DFSC and PDLSCs at passage 3 were characterized by flow cytometry (FACSCan) based on the expression of the accepted markers. In flow cytometric analysis, results indicated positive for CD90, CD166, CD105, CD44, CD13, CD73 cell markers, which are specifically related to stem cells markers, but CD45, CD34 CD38 and CD31 were not observed on hDFSCs or on hPLSCs (Table 1) & (Figure 3 & 4).

Assessment of differentiation to osteoblasts

PDLSCs and DFSCs remained in the osteogenic medium for 14 days, after which, the von Kossa staining confirmed the presence of calcium deposits in the medium (Figure 5).

Assessment of differentiation to adipocytes

PDLSCs and DFSCs remained in the adipogenic medium for 21 days, after which, Oil Red O staining confirmed the presence of intracellular lipid vacuoles (Figure 6).

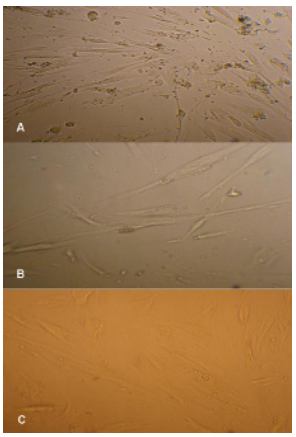

Figure 1. A. First passage of PDLSCs of one sample 1 (×10 magnification). B. First passage of PDLSCs of one sample (×25 magnification). C. Third passage of PDLSCs of one sample, 14th day (×40 magnification).

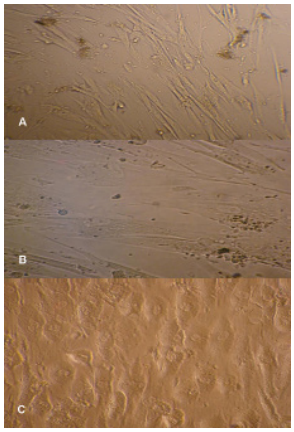

Figure 2. A. First passage of DFSCs of one sample (×10 magnification). B. First passage of DFSCs of one sample (×25 magnification). C. Third passage of DFSCs of one sample, 14th day (×40 magnification).

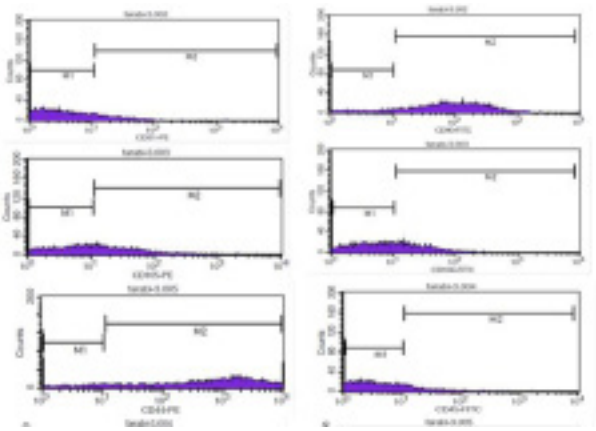

Figure 3. Flow cytometric histograms of PDLSCs (a sample).

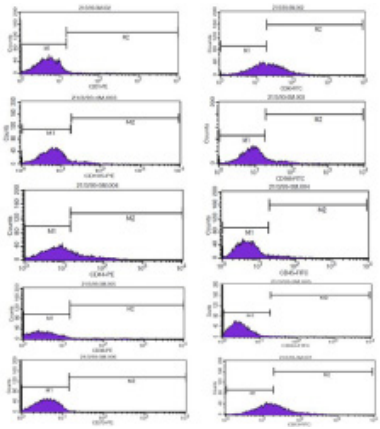

Figure 4. Flow cytometric histograms of DFSCs (a sample).

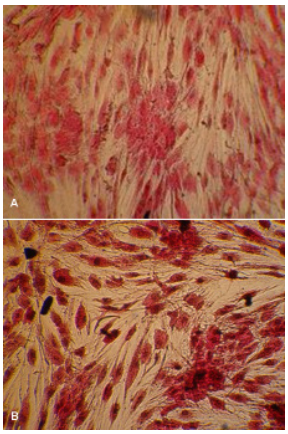

Figure 5. A. Staining of PDLSCs in the osteogenic medium using Von Cossa. B. Staining of DFSCs in the osteogenic medium using Von Cossa.

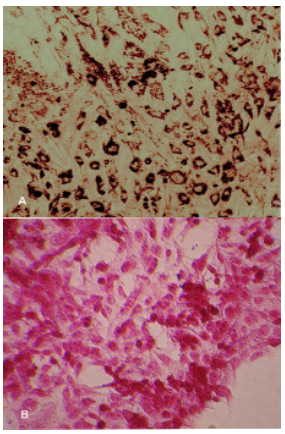

Figure 6. A. Staining of PDLSCs in the adipogenic medium using Oil Red O. B. Staining of DFSCs in the adipogenic medium using Oil Red O.

Marker	Groups	Number	Minimum	Maximum	Mean Fluorescence intensity (MFI)	SD	p-Value
CD90	DFSC	5	4.69	227.56	65.72	91.55	0.42
	PDLSC	5	32.51	157.92	74.47	58.25	
CD31	DFSC	5	3.99	10.13	6.12	2.34	1.00
	PDLSC	5	2.11	8.56	5.86	2.47	
CD166	DFSC	5	10.39	48.71	20.82	15.82	1.00
	PDLSC	5	11.68	18.19	15.10	2.44	
CD105	DFSC	5	5.31	67.09	20.13	26.32	1.00
	PDLSC	5	4.65	26.28	12.15	8.31	
CD45	DFSC	5	6.42	8.38	7.46	0.85	0.55
	PDLSC	5	5.05	11.75	8.49	2.63	
CD44	DFSC	5	13.82	1650.39	341.76	731.54	0.69
	PDLSC	5	13.46	1619.21	417.77	694.10	
CD34	DFSC	5	4.96	24.42	9.90	8.18	0.55
	PDLSC	5	3.84	17.57	11.54	5.38	
CD38	DFSC	5	4.48	10.54	6.75	2.28	0.69
	PDLSC	5	1.87	8.72	5.80	2.82	
CD13	DFSC	5	11.11	135.77	41.86	52.85	1.00
	PDLSC	5	14.79	27.42	20.40	5.11	
CD73	DFSC	5	1.73	5.09	3.35	1.40	1.00
	PDLSC	5	1.64	5.07	3.28	1.23	

Table 1. The rate of CD markers of DFSCs and PDLSCs identified by flow cytometry.

Discussion

During embryogenesis, skin and neurons were derived from epithelial cells in the ectoderm, while DF and PDL tissue was derived from mesenchymal cells. However, there was no particular method used to detect mesenchymal stem cells, but cell behaviors in the culture medium, cells morphology, and cell markers provided enough evidence to recognize stem cells [22, 23]. The most specific method of identifying stem cells remains multi-lineage differentiation [24]. In this study, fibroblastic cells' phenotypes appeared after two weeks of cell culturing, leading to identification of the mesenchymal feature.

Campagnoli et al. (2001) stated that if we want to more definitively identify mesenchymal stem cells, we must search for surface markers such as CD90, CD166, CD105, CD44, CD13, and CD73 on the cell surface, together with the lack of expression of hematopoietic specific markers, such as CD45, CD34, CD38, and CD31 [25]. In several studies, PDLSCs expressed sevs eral cell surface markers, such as STRO-1 (putative marker of stem cells), CD146 (a marker of perivascular cells), STRO-3 (tissue nonspecific ALP), CD13, CD29 (integrin β-1), CD44, CD90 (Thy-1), CD105 (endoglelin), CD106 (VCAM-1), and CD166 (ALCAM) [23, 26-29]. In a study by Navabazam et al. (2013), PDLSCs showed high levels of expression from CD31, CD44, and CD34; however, since CD34 and CD31 are mark-

ers of hematopoietic stem cells, these markers express at low levels in mesenchymal stem cells, similar to the results of this study [30]. Yalvac, Mehmet E., et al [31]. reported that DFSCs were stained positive for CD105, CD90, CD73, CD166, and negative for CD34, CD45, and CD133. According to numerous studies, DFSCs express CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90, CD105 markers and did not express CD34, CD45 markers [32-34].

In this study, flow cytometry tests were performed to verify stem cells. Results showed that CD34 and CD45 were not observed in the cell culture of periodontal tissues; since these markers are hematopoietic markers, this result indicated that these cells were not hematopoietic. Findings were significantly positive for CD90, CD166, CD105, CD44, CD13, and CD73. This result meant that dental follicle and periodontal tissues-derived stem cells were cultured in this study, having the surface marker characteristics of mesenchymal stem cells. This result was in agreement with findings by Chen (2006) and Campagnoli et al. (2001). Both studies accepted that cell markers such as CD44, CD166, and STRO-1 on PDLSCs were from a periodontal ligament source [25, 35].

Conclusion

This study opens the way for further research of human dental follicle and periodontal cells. The present study found that stem cells derived from the PDL and DF express CD90, CD166, CD105, CD44, CD73, and CD13 markers, similar to mesenchymal stem cells. Stem cells have the capacity to differentiate into various cell types according to environmental and micro-environmental inductions. We conducted cell differentiation of the desired cell line proliferation, such as osteoblast cells, which is a good choice for bone and periodontal defect treatments.

Conflict of Interest

There is no conflict of interest to declare.

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