Original Article

Assessment of Surface Markers Derived from Human Periodontal Ligament Stem Cells: An In Vitro Study

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Abstract

Objectives: Periodontal tissue regeneration for treatment of periodontal disease has not yet been mastered in tissue engineering. Stem cells, scaffold, and growth factors are the three main basic components of tissue engineering. Periodontal ligament (PDL) contains stem cells; however, the number, potency and features of these cells have not yet been understood. This study aimed to isolate and characterize the properties of PDL stem cells.

Materials and Methods: In this experimental study, samples were isolated from the PDL of extracted teeth of five patients and then stained immunohistochemically for detection of cell surface markers. Cells were then examined by immuno-flow cytometry for mesenchymal markers as well as for osteogenic and adipogenic differentiation.

Results: The isolated cell population had fibroblast-like morphology and flow cytometry revealed that the mesenchymal surface markers were (means): CD90 (84.55), CD31 (39.97), CD166 (33.77), CD105 (31.19), CD45 (32/44), CD44 (462.11), CD34 (227.33), CD38 (86.94), CD13 (34.52) and CD73 (50.39). The PDL stem cells also differentiated into osteoblasts and adipocytes in osteogenic and adipogenic media, respectively.

Conclusions: PDL stem cells expressed mesenchymal stem cell (MSC) markers and differentiated into osteoblasts and adipocytes in osteogenic and adipogenic media, respectively.

Keywords: Adipocytes; Antigens; Mesenchymal Stromal Cells; Osteoblasts; Periodontal Ligament

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INTRODUCTION

Periodontitis is an infectious and inflammatory oral disease that leads to periodontal tissue destruction and is a major cause of tooth loss [1]. Dentists have succeeded in managing periodontal inflammation through the use of conventional therapies, but have failed to restore the periodontium once it has been damaged. Periodontal regeneration therapy focuses on predictable regeneration of periodontal attachment apparatus, which includes alveolar bone, periodontal ligament (PDL) and cementum. Surgical repair, as opposed to regeneration, is the most common outcome of traditional periodontal treatment [2-5]. Stem cell biology is an important field focused on understanding tissue regeneration and implementation of regenerative medicine [6]. Using tissue regeneration, damaged periodontal tissue can be repaired through the application of stem cells, growth factors and an extracellular matrix scaffold [7]. Stem cells can be divided into two categories: embryonic and adult. Embryonic stem cells are pluripotent and capable of differentiation into nearly any mature cell type. Because of ethical considerations with the use of embryonic stem cells, recent attention has focused on stem cells derived from adult tissues. Adult stem cells have a more restricted differentiation potential compared to embryonic stem cells; however, adult stem cells are able to perform basic functions of these cells, i.e. self-renewal, generation of large numbers of progeny and differentiation into multiple mature cell types [8]. Since the discovery and characterization of multipotent mesenchymal

stem cells (MSCs) derived from the bone marrow, MSC-like populations from other tissues are now characterized according to the 'gold standard' criteria established for bone marrow MSCs [1].

Phenotypically, MSCs express several non-specific markers. CD90 (Thy-1; thymocyte differentiation antigen 1), CD13 (zinc metalloproteinase), CD73 (5'-ribonucleotide phosphohydrolase) and CD105 (endoglin) are markers of MSCs.

CD34 (glycoprotein), CD45 (leukocyte common antigen) and CD31 (clearing aging neutrophils) are used to identify hematopoietic stem cells from MSCs; low level of these markers show MSCs. CD38 (cyclic ADP ribose hydrolase) is present on lymphocytes and natural killer cells and plays a role in cell adhesion and intracellular calcium ion regulation. CD44 plays a role in cell-cell interactions and cell adhesion and migration. This protein is not present on the surface of dendritic cells or platelets. CD166 (a membrane glycoprotein) is an immunoglobulin [9-12]. Experts agree that adult human MSCs do not express CD45, CD34, CD14 or CD11 hematopoietic markers nor do they express CD80, CD86 or CD40 co-stimulatory markers or CD31 (platelet/endothelial cell adhesion molecule or PECAM-1), CD18 (leukocyte function-associated antigen or LFA-1) or CD56 (neuronal cell adhesion molecule-1) adhesion molecules. They can, however, express CD105 (SH2), CD73 (SH3.4), CD44, CD90 (Thy-1), CD71 and Stro-1, as well as the adhesion molecules CD106 (vascular cell adhesion molecule or VCAM-1), CD166 (activated leukocyte cell adhesion molecule or ALCAM), intercellular adhesion molecule (ICAM)-1, and CD29 [13-18]. Potential MSC populations can be identified by assessing their capacity to differentiate into bone, fat or cartilage in vitro [15].

Evidence shows that PDL cell populations can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts). Presence of multiple cell types in PDL suggests that this tissue may have stem cells that can maintain tissue homeostasis and regenerate periodontal tissue [19,20]. Similar to other dental stem cells, PDL stem cells (PDLSCs) exhibit osteogenic, adipogenic, and chondrogenic characteristics under suitable culture conditions [21-23]; therefore, PDLSCs may be an excellent cellular source for PDL regeneration [24]. The purpose of this study was to isolate and characterize human PDLSCs derived from the root, and to investigate their microscopic phenotype, surface marker expression, and differentiation into adipocytes and osteoblasts.

MATERIALS AND METHODS

In this experimental study, participants were referred to the Department of Periodontology, School of Dentistry, Tehran University of Medical Sciences.

The research protocol was reviewed and approved by the Ethics Committee of the Dental Research Center of Tehran University of Medical Sciences (IR.TUMS.REC.1395.2818). All patients signed written informed consent forms. The PDL tissue from the extracted teeth of five patients between 19 and 51 years was cultivated and evaluated via flow cytometry for surface proteins of stem cells.

Teeth were extracted due to orthodontic or prosthetic reasons. The PDL tissue was then obtained from patients, who had no chronic diseases or history of smoking, alcohol consumption or medication use. Included teeth consisted of those extracted for orthodontic or prosthetic treatment purposes, those with no endodontic infections and no lucent or opaque lesions observed on radiographs, and fully erupted teeth from patients who exhibited no periodontal infection and had no history of previous treatment.

Patients rinsed their mouth with 0.2%

chlorhexidine, after which their lips and skin were prepped with diluted Betadine. After induction of anesthesia, atraumatic extraction was performed. Root surfaces were scraped with a back-action chisel and isolated tissue samples were placed in a nutrient solution for a minimum of 20 minutes at 4°C, avoiding contact with the walls of the dish. The solution, which contained 15mL of Roswell Park Memorial Institute medium combined with penicillin, streptomycin, gentamicin and amphotericin B, was then transferred to the Laboratory of Stem Cells of Farabi Hospital within three hours.

Cell isolation and culture: In sterile conditions under a hood, samples were rinsed several times with phosphate-buffered saline (PBS) containing antibiotics. They were then immersed in a tissue solvent solution containing Dulbecco's modified Eagle's medium (DMEM) and collagenase type 1 at a concentration of 250 u/mL in a shaking incubator at 37°C for one to two hours. The solution containing the sample was centrifuged using 5810R Eppendorf centrifuge for five minutes at 1500 rpm in order to pellet the cells. The cells were then cultured at 37°C in presence of 95% oxygen and 5% carbon dioxide [25, 26] each in T75 flasks containing DMEM and 10% fetal calf serum (FCS). The medium was replaced after 48 hours and refreshed every four days thereafter.

The media contained the following ingredients:
DMEM+100 mg/mL penicillin+ 100 mg/mL streptomycin+10% fetal bovine serum (FBS)+5 mg/mL amphotericin B+0.1% L-glutamine.

When 80% of the flask was covered by cells, indicating an 80% confluence, cells were detached using 0.2% trypsin, neutralized with FBS, and passaged at 1:3 ratio.

Fluorescence-activated cell sorting: luorescence-activated cell sorting in flow cytometry is a technique used for counting and examining microscopic particles such as chromosomes and cells. Test particles are placed into two or more containers and suspended in a fluid at a rate of approximately 5 to 50 meters per second through a narrow opening, and from the opposite narrow beam of laser light passes, and enables data collection from 5,000 to 50,000 cells per second [27].

Passage of cells (subculture): Tissues obtained by biopsy were cultured and, after passage, the cells were proliferated and sub-cultured in new media in order to increase their count. After 4/5 of the flasks was covered by cells, the cells were removed from the flask and 1cc of ethylene diamine tetra-acetic acid solution was added. Likewise, this solution was then removed and 1cc of 0.25% trypsin was added to detach the cells from the bottom of the flask. After detachment, FBS was used to neutralize trypsin. The sample was centrifuged again and placed in DMEM, and further passage was performed after 14 days.

Counting the cells: In order to count the cells, 10µL of the sample was placed on a slide and cells were counted without staining under an inverted microscope at ×10, ×20 and ×40 magnifications. Each flask housed 2×10^6 cells [28].

Evaluation of surface markers by flow cytometry: Flow cytometry was used to assess the presence and extent of surface markers in the extracted tissue. An antibody that binds to fluorescein was poured onto a single cell suspension and was then conjugated to markers with labeled antibodies. Fluorescent-iso-thio-cyanate and phycoerythrin dyes were employed in this process, and 10 cell surface markers were examined namely CD13, CD31, CD34, CD38, CD44, CD45, CD73, CD90, CD105, and CD166.

To prepare the suspension of individual particles stained similarly in each of the PDL stem cell samples in the third passage, 0.25% trypsin/ethylene diamine tetra-acetic acid was added and cells were counted. Approximately, 10^4 cells were collected from each sample and placed in separate tubes. The tubes were then placed in an incubator on a rocker rotator in order

to suspend cells evenly. Tubes were centrifuged at 1000 rpm for six minutes and 3% human sera were added, after which the tubes were incubated for 30 minutes at room temperature. Contents of the tubes were centrifuged again at 1000 rpm for six minutes and PBS was added. The cells were treated with anti-CD13, anti-CD31, anti-CD34, anti-CD38, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti-CD105 and anti-CD166, and after washing, the cells were placed in 100μL of 1% paraformaldehyde and studied using flow cytometry.

Differentiation to osteoblasts: Two samples of third passage PDLSCs 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: Two samples of third passage PDLSCs 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 process, the samples were washed with PBS and fixed in 4% paraformaldehyde in PBS followed by a final rinse with distilled water. After three weeks, they were stained with 0.5% Oil Red O [29-32].

RESULTS

Microscopic evaluation: During microscopic observation, samples from the isolated and cultivated PDLSCs showed polyhedral elongated cells with an oval nucleus at the center of the cell. The cells were attached to the flask.

Flow cytometric assessment: Expression of CD13, CD73, CD90, CD105, CD166, CD44, CD38, CD34, CD45, and CD31 surface markers was examined on third passage PDLSCs, and the results of the flow cytometric analysis are illustrated in Table 1.

The results of flow cytometric analysis in this study showed that CD90 was expressed by 77.6% with a mean frequency of 84.5, CD31 by 7% with a mean frequency of 39.9, CD166 by 25.88% with a mean frequency of 33.77, CD105 by 12.64% with a mean frequency of 31.1, CD45 by 14.45% with a mean frequency of 32.4, CD44 by 24.47% with a mean frequency of 462.1, CD34 by 4.1% with a mean frequency of 227.3, CD38 by 4.36% with a mean frequency of 86.9, CD13 by 48.78% with a mean frequency of 34.5, and CD73 by 1.19% with a mean frequency of 50.3.

Assessment of differentiation to osteoblasts: The cells remained in the osteogenic medium for 14 days, after which, the von Kossa staining confirmed the presence of calcium deposits in the medium.

Table 1: Frequency of surface markers expressed in the samples (n=5)

Markers	Mean	Standard deviation	Maximum	Minimum
CD90	84.55	60.56	173.49	39.52
CD31	39.97	29.73	88.05	9.24
CD166	33.77	6.53	45.02	28.56
CD105	31.19	21.02	54.75	5.84
CD45	32.44	11.58	49.55	17.24
CD44	462.11	715.08	1699.65	34.27
CD34	227.33	179.84	428.07	36.83
CD38	86.94	81.98	212.34	10.51
CD13	34.52	9.45	47.29	22.20
CD73	50.39	58.61	154.53	12.74

Assessment of differentiation to adipocytes: The cells remained in the adipogenic medium for 21 days, after which, Oil Red O staining confirmed the presence of intracellular lipid vacuoles.

DISCUSSION

For the purpose of this study, PDL samples were obtained by scratching the root's surface, a process which was previously used by Navabazam et al, [29] Park et al, [30] and Silverio et al, [31] in order to sample the PDL. Wang et al, [32] compared PDL stem cells of tooth roots (r-PDLSCs) and PDL stem cells of the alveolar bone (a-PDLSCs) and found that a-PDLSCs had higher differentiation potential in osteogenic and adipogenic media. Their study also revealed that a-PDLSCs expressed higher stem cell markers and higher markers associated with mineralization compared to r-PDLSCs. Moreover, a-PDLSCs showed higher alkaline phosphatase activity and more complete alveolar bone reconstruction in specific media, and a-PDLSCs and r-PDLSCs had synergistic effects on periodontal regeneration.

The present study used healthy PDL without inflammation. In a study by Park et al, [30] proliferative and differentiating potential of healthy PDLSCs and inflamed PDLSCs showed no difference, while the migration capacity of inflamed PDLSCs (PDLSCs from inflamed tissues of intra bony defects) increased significantly. Both groups (healthy PDLSCs and inflamed PDLSCs) showed STRO-1, CD146, CD90, and CD44 surface markers while showing no signs of CD19 marker. Both groups also expressed high levels of periostin protein. These results were consistent with those of Tang et al, [33] and Zheng et al, [34] which compared the properties of stem cells derived from inflamed and healthy PDL. Similar to the results of Park et al, [30] the present study showed expression of CD44 and CD90 markers and periostin protein, and samples differentiated successfully in osteogenic and adipogenic media.

In the current study, similar to the studies conducted by Navabazam et al, [29] and Silverio et al, [31] samples were cultured in DMEM, which has higher calcium and magnesium ions than Roswell Park Memorial Institute medium and is particularly suitable for cell growth. This medium contained 100 units/mL of penicillin to prevent the growth of gram-positive bacteria, 100 µg/L of streptomycin to inhibit the growth of Gram-negative bacteria, 50 µg/mL gentamycin to prevent the growth of Gram-negative and Gram-positive bacteria and amphotericin B antifungal drug.

In a previous study, Suchanek et al, [35] cultured third molar pulp cells in three media and assessed the phenotypic and biological characteristics of dental pulp stem cells (DPSCs). The study used α- minimum essential medium (MEM) containing 2% FCS, α-MEM containing 10% FCS, α-MEM containing 2% FCS, and an insulin-transferrin-selenium supplement to increase the proliferative activity of DPSCs. Results showed that cell size ranged from 15 to 16µm and the viability of cells was above 90% in the ninth passage. Phenotypic analysis of cells was positive for CD29, CD44, CD90, and HLAI markers and negative for CD34, CD45, CD71 and HLAII markers. No signs of degeneration or spontaneous differentiation were observed.

The expression of CD34, CD31 and CD45 in our study was inconsistent with previous studies [8, 36]. CD34, CD31 and CD45 are markers of hematopoietic stem cells, which were slightly expressed in our study samples. A high concentration of CD34 marker was observed as well, and high levels of expression of this marker were seen on the surface of a small number of cells. When PDLSCs from healthy and inflamed samples were examined in a study by Park et al, [30] only CD90 and CD44 markers were expressed at high levels, consistent with the present study. In a study by Navabazam et al, [29] PDLSCs showed high levels of expression of CD31, CD44, and CD34; however, since CD34 and CD31 are markers of hematopoietic stem cells, it is expected that these markers express at low levels in MSCs, similar to the present study. In studies regarding DPSCs and stem cells from human exfoliated deciduous

teeth [8,35,36], the CD90 marker showed a high expression rate of 80%, while the highest level allocated to this marker in the present study was 77.6%. According to several studies, PDLSCs express several cell surface markers, namely 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 (endoglin), CD106 (VCAM-1), and CD166 (ALCAM) [37-41]. In the present study, expression of CD90, CD166, CD105, CD44, and CD13 was observed at moderate and high levels. One of the criteria set by the International Society for Cellular Therapy for human MSCs is the ability to differentiate into adipocytes and osteoblasts in vitro. In the present study, two samples were placed in osteogenic media and two were placed in standard adipogenic media in accordance with standard differentiation protocol. Each sample differentiated into osteoblasts after 14 days or into adipocytes after 21 days. Results of this process confirmed that these cells were MSCs and could differentiate into more specialized cells, making them useful for differentiation in tissue engineering under suitable conditions.

CONCLUSION

Our study suggests that PDLSCs express CD13, CD73, CD90, CD105, CD166, CD44, CD38, CD34, CD45, and CD31 surface markers, similar to stem cells in other tissues. In addition, PDLSCs showed differentiation potential in osteogenic and adipogenic media; therefore, these cells may be used as a source of MSCs for growth and regeneration of periodontal tissues in bioengineering. Further research is suggested to be done on PDLSC gene expression to enhance identification of these cells.

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