Isolation and investigation of multipotent human periodontal tissue stem cells

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Abstract

Aim: culture of human preiodontal mesenchymal stem cells to production osteoblast. This might be used for repair of human periodontal defects in future.

Method: periodontal tissues were obtained from periodontium of patients who were candidate for periodontal surgery. They were 25-45 years old and had no systemic diseases, no smoking, and no drug treatment. Tissues were cultured in DMEM medium. Cells were by subsequently expanded by passages. 3 passages were done. Then cells were evaluated by inverted microscope and flowcytometry. We stained PDLstem cells with these markers: CD44, CD90 CD166,CD13, CD34,CD45 .

Finding: PDL stem cells expressed MCSCs markers as shown in flowcytometry. The cells were negative by CD34 and CD45 markers and were positive by CD90, CD166, CD13, and CD44 markers .We saw a monolayer attached cells on the floor of flask macroscopically and we saw spindle cells by inverted microscope. In the microscopic finding we saw nuclear red calcified view with Alizarine staining in day 14th of culture.

Conclusion: Our findings show that human PDL contains a population of multypotent postnatal stem cells can be isolated and expanded in vitro. It provides a reservoir of stem cells from an accessible tissue resource. These cells have capacity of proliferation ex vivo. Therefore tissue regeneration mediated by human PDL stem cells might have potency of practical cellular- based treatment of periodontal defects.

The dental attachment apparatus consists of two mineralized tissues: cementum and alveolar bone, with a soft connective tissue which is embedded between the cementum and the inner wall of the alveolar bone socket, to sustain and help constrain teeth within the jaw.

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Periodontal ligament (PDL) not only has a significant role in supporting teeth, but also contributes to tooth nutrition, homoeostasis and repair of damaged tissues. [1-2]

The continuous regeneration of PDL is thought to involve mesenchymal progenitors arising from dental follicle. It is thus obvious that PDL itself contains progenitors, which can be activated to self-renew and regenerate periodontal tissues such as cementum and alveolar bone.[3] PDL contains heterogeneous cell populations that can be discriminated into either cementoblasts or osteoblasts.[4-5] PDL contains STRO-1 positive cells that maintain certain plasticity, since they can adopt adipogenic, osteogenic and chondrogenic phenotypes in vitro. [6]

A stem cell (SC) is defined as a cell which can continuously produce unaltered daughters, and furthermore, has the ability to generate cells with different and more restricted properties. Pluripotent stem cells can be isolated from the embryonic inner cell mass. However, ethical concerns about the requirement of human embryos and the potential danger of teratoma formation have contributed to the limited use of these cells. [3, 7-8]

When stem cells were first discovered, scientists generally accepted that the differentiation potential of adult stem cells was restricted to the tissues in which they resided. Later, more and more evidence supported that they could even be differentiated into specific cell types of non-related tissues. This property is called "plasticity" or "transdifferentiation". Stem cells can be derived from various tissues. The choice of the tissue source is governed by availability, as well as by the degree of characterization associated with the stem/progenitor cells in term of surface markers and differentiation pathway. [9-10]

Periodontal diseases constitute the most common oral infections in humans and the major cause of tooth loss in adults. [11-12] The reconstruction of healthy periodontium destroyed by periodontal diseases is a basic goal of periodontal therapy.

Tissue engineering procedures have been developed for many tissues and organs such as bone, heart, liver and kidney. In the recent years, researches have also isolated cells from postnatal dental pulp, and found that these populations exhibited the two basic properties of stem cells: 1) self-renewal, and 2) multilineage differentiation. This fact provides the possibility to repair damaged teeth with the patient's own tissue, when dental tissue engineering procedure can be developed.[13-15] In the present study, we report the isolation and characterization of a unique stem-cell population from PDL tissue.

Materials and methods

Patient selection

Ten Iranian patients from 25 - 45 years old, 4 men and 6 women, undergoing periodontal surgical referred to the Periodontal Clinic of the Tehran University of Medical Sciences participated in the study. Informed consent was obtained from all participants with the signed form that was previously reviewed and approved by the patients. Patients completed personal and familial medical and dental history questionnaires; exclusion criteria were a history of smoking, alcohol user and any medication. Periodontal tissues were gained from the surgically excisional tissues routinely separated from dental roots by periodontist during the periodontal surgery using a sterile scalpel. these patients had periodontal destruction and they had need periodontal surgery.

These tissues were translating by solution DMEM (Dulbecco's modified Eagles medium) in 30 minutes to Iranian Tissue Bank Research & Preparation Center of Imam Khomeini 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 phosphate-buffered saline (PBS, Sigma, St. Louis, MO) then centrifuged for 10 minutes at 1500 rpm and cut into fine pieces. These pieces were digested with 0.1% collagenase IV (Nitta Gelatin, Osaka, Japan) for 1 hours, centrifuged for 10 minutes at 1500 rpm again ,and were shaken vigorously at 37°C for 15 minutes. The resultant cell suspension was filtered through a 70 µm nylon mesh to remove tissue debris. Subsequently, collagenase was removed through dilution with solution PBS and centrifuged twice for 5 minutes at 1500 rpm. Unfortunately, 4 specimens were failed due to fungus contamination, so DMEM medium supplemented by Amphotericine B.

1 × 103 deposited cells were plated into 25 cm2 flask contains Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented of low glucose DMEM [Sigma, USA], 10% FBS, glutamine-L, 100 U penicillin, 100 mg/ml streptomycin, 5 mg/ml amphotrycine B, and 0.05% EDTA for passage(figure-1).

Until 72 hours every 3hours microscopic assay was done (figure-2).The culture medium was changed after 24 hours and then every 4 days. When 4/5 of the bottom of the flask covered by cells, the cell passage was done using 0.25% trypcine

containing 1 mM EDTA (Gibco BRL), and passaged at 80% confluency .The cells incubated at 37˚C with 5% CO2. The cells of 2 specimens proliferated and proliferating of 4 specimences were failed.

Figure-1:flask for culture

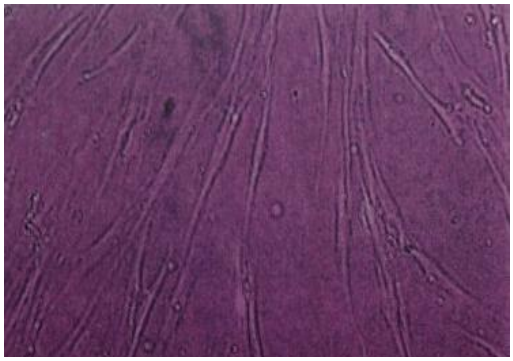


Figure-2: Microscopic features of PDLScs observed every 3 hours in the first 3 days of culture. elongated polyglonal cells are seen (20magnification)

Identification of Stem cells by flowcytometric analysis

Fluorescence activated cell sorter (FACS) was performed to analyze the surface markers of isolated human PLSCs (hPLSCs). In brief, the cells were treated and counted using 0.25% EDTA- Trypcine and neobar lam and centrifuged. 105 to 106 cells into 2 units of 3% FBS make the culture medium suitable for adding monoclonal antibody and it was placed at room temperature for 30 min. Again, they 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 again PBS and were conjugated with anti CD166, anti CD105, anti CD45, anti CD34, and anti CD90 [FITC].The resultant mixture was placed at 4˚C out of light for 45 minutes and after washing were put in the flowcytometry device.

Differentiation of PDLSCs into osteoblasts:

After the isolation of PDLSCs, they were placed in DMEM plus 10% FBS, including 10 mM β-glycerol phosphate, 10-7 M dexamethasone, and 50 mg/ml ascorbic acid. For analyzing osteoblast differentiation, alizarin red staining technique was used, which is a qualitative analysis. Then, the samples were observed under a Digital inverted microscope (Cetti, Spain) (figure-3).

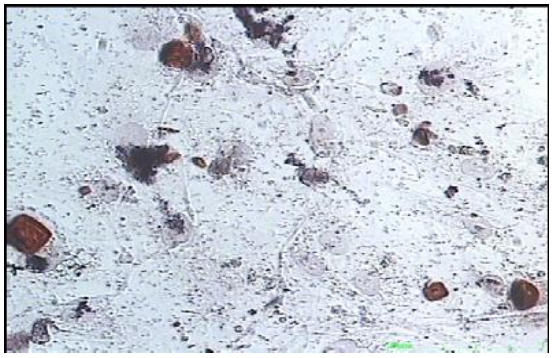


Figure-3: Alizarine red staining stem cells after 2 weeks culture

Finding

Human PDLSCs at passage 3 were characterized by flow cytometry (FACSCan) based on the expression of the accepted markers. In flowcytometric analysis, results indicated positive for CD90 (59%), and CD44 (96%) cell markers, which are specifically related to stem cells markers (figure 4-5), but CD45 and CD34 were not

observed on hPLSCs. The expressions of all the studies were similar for the entire expansion period and for all the samples utilized in the experiment: on the average 66 ± 48% of human PDLSCs expressed CD13, 65 ± 13% expressed CD90, 59 ± 5% expressed CD166, and 70 ± 1% expressed CD44. (Table 1)

	N	Minimum	Maximum	Mean	Std. Deviation
CD13	**2**	**65.66**	**66.34**	**66**	**0.48**
CD90	**2**	**55.16**	**74.56**	**64.86**	**13.71**
CD166	**2**	**55.08**	**62.71**	**59.89**	**5.39**
CD44	**2**	**69.31**	**71.17**	**70.24**	**1.31**
CD34	**2**	**82.42**	**97.20**	**89.81**	**10.45**
CD45	**2**	**91.60**	**92.85**	**92.22**	**0.88**

Table 1: The rate of CD markers of stem cells identified by flowcytometry

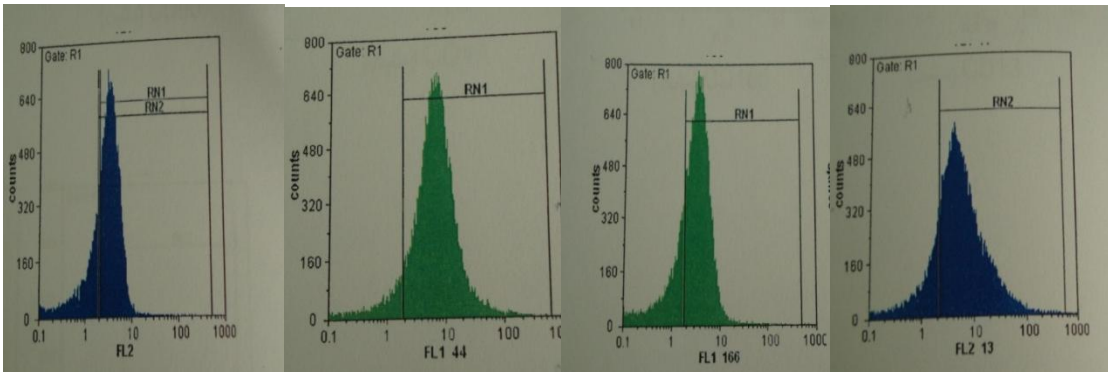
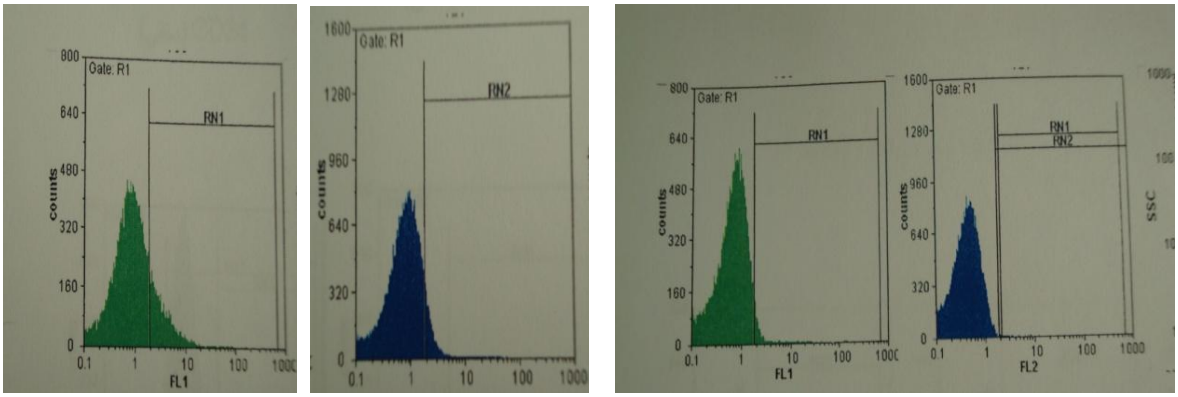


Figure-4: Diagrammatic positive results of flowcytometric analysis for CD90 (R2), CD44 ,CD166 ,CD13



A-control solution B-control solution C-Stem cell solution

Figure-5:Diagramatic negative results of flowcytometric analysis (c) for CD34 (R1)and CD45(R2) and control solution(A and B)

Discussion

During embryogenesis, skin and neurons are derived from epithelial cells in the ectoderm, while PDL tissue is derived from mesenchymal cells. However there isn't any particular method to detect mesenchymal Stem cells, but cell behaviors in the culture medium, cells morphology, and cell markers can provide enough evidences to recognize stem cells(16-17). The most specific way of proving stem cells is Multilineage differentiation(6). In this study fibroblastic cells phenotype was appeared after two weeks of cells culture leading to mesenchymal feature. Although 8 specimens of 10 cases were failed to reach to end of passage because of fungal infections but 2 specimens were cultured successfully. In a study by capagnoli (18) it has been stated that if we want to more definitively identify mesenchymal stem cells, we must search for surface markers such as CD105, CD166, CD90, and CD44 on the cell surface together with lack of expression of hematopoietic specific markers like CD34, CD45 and CD14 (18). In this study, flowcytometry test was done to verify stem cells. Results showed that CD34 and CD45 were not observed in the cell culture of periodontal tissues derived while these markers are hematopoietic markers, and this indicated these cells were not hematopoietic. Findings indicated significantly positive for CD166, CD13, CD44, and CD90. This result means periodontal tissues-derived stem cells were cultured in this study have the surface marker characteristics of mesenchymal stem cells which is in agreement with findings by Chen(19) and Capagnoli 1(18)studies. They accepted cell markers such as CD44, CD166, and STRO-1 on PDLSCs are from periodontal ligament source.

In this survey the cells incubated in the osteogenic medium and then stained with alizarin e red and were studied by microscope. In this study PDLSCS showed calcifying nuclear area by day 14 in the microscopic assay. It seems there was some proteins like bone morphogenic protein 4 in the culture induced Calcifying behavior of PDLSCS culture. This phenomena is observed by day 21 1n Gay study(6).

Moreover, CD29, CD13, CD90 (17), CD44, and CD166(20) were expressed in PDL cells. Park J-C et al (21) demonstrate inflamed human PDL stem cells(ihPDLSCs) could be successfully isolate from inflamed PDL and characterize as MSCs.

Conclusion

This study can open the way for further studies of human periodontal cells. Undifferentiated cells have capacity to differentiate to various cell types according to the environmental and micro environmental inductions, and by controlling the environmental inductions and present signals. We can conduct cell differentiation to the desired cell line proliferation like osteoblst cells, which is a good choice for bone and periodontal defects treatment.

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