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Methods for Proliferation, Differentiation and Collagen Production Studies of PDL Stem Cells

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Abstract: ***Human periodontal ligament (PDL) is a promising source of mesenchymal stem cells (MSC), as these cells could serve as a therapeutic tool for various pathological conditions. For this purpose methods for PDL stem cells isolation, cultivation and guided differentiation have to be broadly promoted and utilized.***

Keywords: periodontal ligament, stem cells, markers, differentiation

1. Introduction

Current tissue engineering offers promising methods for periodontal regeneration, based on the concept of promoting repopulation of the wound area with cells from the periodontal ligament (PDL). Presence of stem cells within human PDL is revealed by Seo et al. in 2004 0. PDL stem cells are a type of mesenchymal stem cells (MSC) usually obtained from routinely extracted impacted/partially impacted third molars or premolars extracted for orthodontic reasons, as they have been demonstrated to possess all typical characteristics and regenerative potency of the MSC 0[2][3][4]. These cells are capable in appropriate conditions to proliferate, synthesize extracellular matrix and differentiate into various cell lineages including osteogenic, adipogenic, chondrogenic, neurogenic, etc. Stem cells are generally cultivated in culture medium containing calf serum. However, investigators recommend the use of serum-free media in order to avoid the presence of animal products, as it represents a huge obstacle for application of *in vi*tro cell therapy methods in clinical practice [5][6].

Various methods have been proposed for PDL stem cells isolation, cultivation and characterization. Wide range of investigation has been conducted to reveal the effects of different growth factors on the cell properties. The aim of the present article is to summarize and present some of the most useful techniques for *in vitro* experimental work with human PDL MSC.

2. Methods

2.1 PDL cell cultures obtained from permanent teeth

The method for PDL stem cells isolation and cultivation was firstly described by Seo et al. 0. The disease free impacted wisdom teeth are obtained from individuals with good general health, usually at age between 18 and 40 years old. PDL cells are isolated by scraping with sterile scalpel blades from the middle third part of the root (Figure 1, 2), followed by enzymatic digestion with 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 h at 37^{o}C. Single-cell suspensions are obtained by passing the cells through 70 µm strainer. Tissue explants are maintained in alpha-minimal essential medium (alpha-MEM) or Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotic-antimycotic and 10-15-20% heat inactivated fetal bovine serum (FBS). The culture medium is refreshed every 2nd or 3rd day. Cells migrate from the explants and within the next 2-4 weeks they become 85-90% confluent as determined by phase contrast microscopy (Figure 3). For cell cultures multiplication sub-confluent cell layers are detached via trypsinization (0,05% trypsin/EDTA) and transferred to new culture dishes/flasks. Culture flasks should be monitored daily, as any contaminated flasks are immediately removed.

Some authors apply the overnight digestion method for PDL stem cells isolation. Tissue explants are finely chopped and cells are released by overnight incubation with high glucose DMEM supplemented with 10% FBS and 1% antibiotic, 1 mg/ml collagenase and 0,6 mg/ml protease at 37^{o}C [7].

Another technique for PDL stem cells isolation is the outgrowth method. PDL is collected from the middle third of the root and rinsed 5 times with alpha-MEM containing FBS and antibiotic. After that the tissue is carefully cut on pieces and placed in a culture dish with minimal amount of culture medium to avoid tissue floating. Culture medium is replaced every 3rd day. When reaching sub-confluence, cells are passaged using 0,25% trypsin-0,1% EDTA solution [3][8]. Tanaka et al. [8] revealed that PDL stem cells demonstrate different characteristics based on the isolation method: enzymatic digestion leads to a higher proliferation rate and solid MSC properties, while the outgrowth method is associated with fibroblast-like properties.

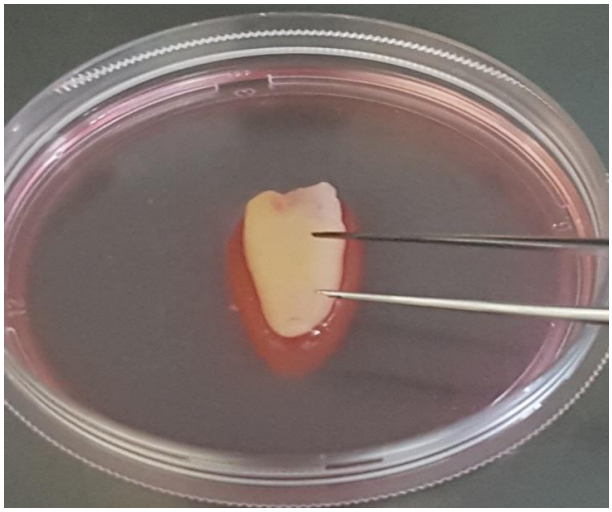

Figure 1: Extracted human premolar for orthodontic reasons. PDL stem cells are isolated from the middle third of the root.

Figure 2: Extracted human wisdom tooth, followed by PDL scraping with sterile scalpel blade

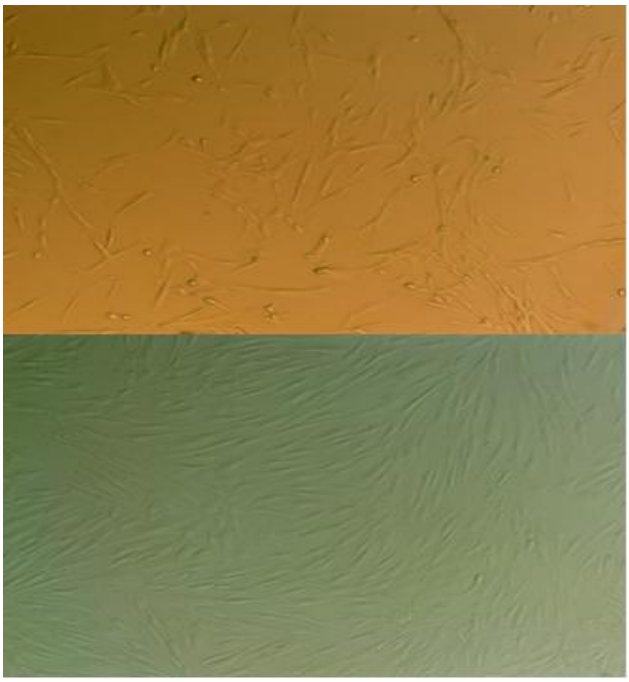

Figure 3: Isolated human PDL stem cells

2.2 Cell proliferation assay

Cell proliferation is crucial for the tissue regeneration. MSC are capable of dividing and renewing themselves for long periods and thus they provide huge number of unspecialized cells that can give rise to various cell lineages. Different *in vitro* methods are used to estimate the ability of stem cells to proliferate in certain conditions. Cells are seeded onto culture dishes and incubated in for 24h, 48h, 3 days, 1 week, etc. in accordance with the experimental design. After that cells are detached by adding trypsin–EDTA at an indicated time point and counted via hemacytometer after Trypan Blue staining [8]. This is one of the most frequently applied cell proliferation tests.

Cell proliferation could be measured using bromodeoxyuridine (BrdU) cell proliferation kits. According to the methods of Zheng et al. [9] BrdU-labeling reagent should be added to the cell cultures. After washing with Phosphate Buffered saline (PBS), the absorbance of the incorporated BrdU is measured via microplate spectrophotometer.

Various kits are used to assess cell proliferation (CytellTM Cell Cycle Kit (GE Healthcare); CyQUANT Cell proliferation Assay Kit (Molecular Probes; Invitrogen); Cell Counting Kit-8 (Dojindo, Kumamoto, Japan); etc.) according the manufacturers' instructions. The cell number evaluation is usually based on the amount of the total DNA in the sample.

2.3 PDL stem cell characterization

Effective techniques are developed to prove the presence of MSC within human PDL. Two of the most useful methods are flow cytometry and immunofluorescence, based on specific antibodies labeling to cell surface markers. The positive expression of STRO-1 and CD146 markers has been reported to characterize PDL stem cells 0[10]. The expression of additional stem cell markers, including CD105, CD117, CD44, CD49f, etc. is also indicative for presence of MSC in the tissues.

2.4 Flow cytometry analysis

Flow cytometry is a quantitative method that can be applied for MSC characterization and separation. Cells are prepared as single cell suspension by trypsinization (trypsin/EDTA digestion) and harvest immediately after detachment. PDL cells are then washed with PBS and incubated with primary non-conjugated antibodies specific for cell surface markers or isotype control antibodies for approximately 30 min at 4^{o}C or at room temperature (depending on the experimental design). After washing with PBS cells are incubated with secondary fluorescent antibodies for 10 to 30 min in the dark. Isotype antibodies serve as a control. Antibodies directly conjugated with fluorochromes can be used to simplify the procedure: after PBS washing cells are incubated with fluorescent conjugated antibodies in the dark for 10 to 30 min. Labeled cells are analyzed via flow cytometer. Intracellular and superficial markers expression is often assessed via permeabilization kits-Perfix kit (Beckman Coulter International SA, Nyon, Switzerland)

following the manufacturer's instructions. Excess antibody is removed by washing. Overall, the surface and intracellular markers analysis proved the mesenchymal origin of the cells and their ability to differentiate into various cell types when cultured in appropriate conditions.

2.5 Immunofluorescence

Immunofluorescence is a qualitative method. We recommend a protocol used by many scientists with slight modifications [7][11]. Cells are fixed with 4% paraformaldehyde in PBS at room temperature at pH-7,2 or ice-cold methanol at -20^{o}C for 20-30 min. Immediately after that cells were washed 3 times with PBS and incubated with 1% BSA-PBS for another 30 min, followed by permeabilization with 0,05% Tween20 for 10 min and with 0,05% Triton X-100 for 30 min. In the next step, cultures were processed for Immunofluorescent staining. Expression of the stem cell and differentiation markers can be assessed.

Primary monoclonal non-conjugated antibodies are used, followed by incubation with conjugated fluorescent secondary antibody. Incubation time is 1h in the dark for both-primary and secondary antibodies with washing step (3 times with PBS) in-between. When fluorescent conjugated antibodies are applied, cells are incubated for 1h protected from light.

Following 3 washes with PBS, DAPI (4,6-diamidino-2-phenylindole) is added to the cells for 15 min to stain the nuclei. After the final wash cells are visualized using inverted fluorescent microscope. Images are collected from a few randomly chosen fields in the culture dish, followed by accurate analysis (Figure 4).

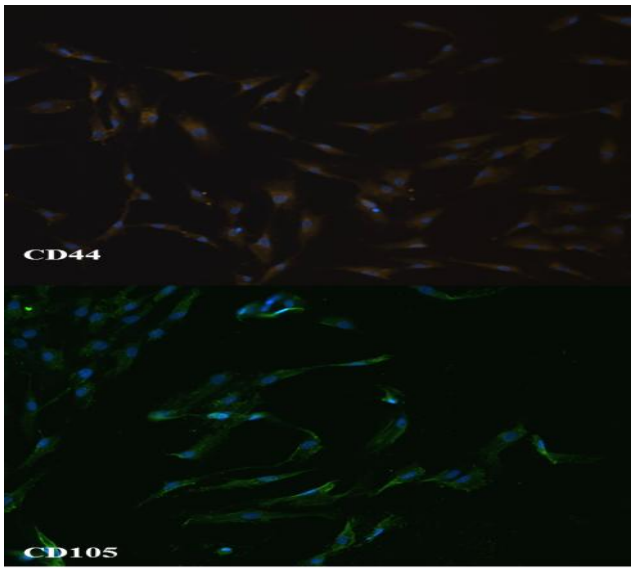

Figure 4: Stem cell markers expression in human PDL cell culture; orange and green-markers expression, blue-nuclei staining

2.6 Collagen synthesis analysis

Collagen is the major protein component in the extracellular matrix (ECM) of human PDL. Total soluble collagen production and expression of specific collagen markers could be assessed via various functional tests, flow cytometry and immunofluorescence techniques. Wide range of studies report the amount of total collagen released in the supernatant or cumulated on the cell layer using the SircolTM Collagen Assay kit (Biocolor Ltd, Carrick Fergus, United Kingdom) according to the manufacturer's instructions [12][13]. At first, standard curve is determined by placing serial dilutions of collagen reference in 1.5-ml tubes. Cells were incubated in experimental culture conditions and cell supernatants are collected. A 100-µL aliquot of the supernatant was added to 1mL of the dye reagent (a component of the kit) in plastic tubes provided with the kit. The tubes were placed in a gentle mechanical shaker for 30 min at a room temperature. After centrifugation at 12 000 rpm for 10 min, the supernatants were discarded and the pellets were dissolved in 1 mL of the alkali reagent (provided with the kit). Acid-soluble collagen is extracted from the cell layer after incubation with 0.5 M acetic acid containing 0,1 mg/ml pepsin for 2h at room temperature or at 4^{o}C overnight. Two hundred µL of each sample were transferred to individual well in 96-well plate once the pellets were completely dissolved and the relative absorbance is measured with microplate reader.

Collagen expression can be evaluated through immunofluorescence and flow cytometry analysis using the abovementioned protocols. Either appropriate primary non-conjugated in combination with secondary fluorescent antibodies, or antibodies conjugated with fluorochromes diluted in BSA-PBS are recommended for these purposes.

2.7 Osteogenic differentiation

Osteogenic differentiation in cell culture is usually assessed via quantitative Alizarin Red S method recommended by Gregory et al. [14], after 3-5 weeks incubation. Similar method is von Kossa staining. Both techniques are able to detect calcium compounds deposited in the ECM as previously described [15][16]. Prior staining, cells were washed with distilled water and fixed with 70% ethanol for 30 min or with 10% buffered formalin for 10 min at room temperature. Two percent Alizarin Red S solution with pH 4,2 was placed in each well for 5 min, followed by 3 times washing with PBS. The dye intensity was determined by absorbance measurement on a microplate reader. In addition, mineral nodules formation was detected via von Kossa staining following fixation with 10% buffered formalin, washing with deionized water and incubation with 1% silver nitrate under UV light for 45 min. The next step is adding of sodium thiosulfate to fix the positive dark staining.

3. Conclusion

The aim of the present article is to report some of the most broadly used methods for PDL stem cells isolation and characterization. The presence of non-differentiated cells in dental tissues is an excellent perspective for regenerative medicine as various techniques for cell isolation, multiplication and differentiation should be established in the daily practice.

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