Isolation and in vitro Characterization of Mesenchymal Stem Cells Derived from the Pulp Tissue of Human Third Molar Tooth

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Abstract

Background: It is still controversial that the stem cells isolated from human dental pulp meets the criteria for mesenchymal stem cells (MSCs). The aim of the present study was to examine whether or not they are MSCs, or are distinct stem cells population residing in tooth pulp.

Methods: Adherent fibroblastic cells in the culture of pulp tissue from human third molars were propagated through several successive subcultures. Passaged-3 cells with a tendency to differentiate into odontoblastic cells were used to examine the key properties of MSCs including typical tripotent differentiation potential into bone, cartilage and adipose cell lineages and the expression of typical surface antigens. Moreover, they were examined for growth capacity in culture.

Results: Dental pulp stem cells successfully progressed towards differentiation among three skeletal cell lineages. More than 90% of the cell population exhibited the expression of surface antigens known to be found on mesenchymal lineages such as CD105, CD90, CD44, and CD73, while only less than 2% expressed endothelial-hematopoietic epitopes including CD56, CD11b, CD34, CD31, CD33, and CD45. The cells exhibited a relatively high proliferation capacity with population doubling time of about 21.9 hours.

Conclusion: The dental pulp stem cells are of MSC population, and may be considered suitable for use in regenerative medicine, owing to their relatively rapid rate of in vitro propagation.


Keywords • Dental pulp • odontoblast • mesenchymal stem cells • proliferation

Introduction

Mesenchymal stem cells (MSCs) are a promising tool for cell-based treatment of variety of tissue defects due to their capacity of extensive replication and potential of multilineage differentiation. These cells were first isolated and described from bone marrow as a colonogenic fibroblastic cell population capable of producing deposits of bone and cartilage-like tissues in culture.¹ Due to the disadvantages associated with marrow MSCs including the burden of marrow harvest, the limited number of MSCs in marrow tissue and in vitro ageing problems
of the cells, many attempts have been made in
the search for other sources of MSCs. In this
regards, several tissues including adipose, cord
blood, peripheral blood, amniotic fluid, cartilage
and bone have been shown to contain MSCs.
Dental pulp is another exciting tissue that
may contain MSCs. This tissue has been the
subject of a number of investigations attempting
to isolate and characterize stem cell popula-
tions. The evidences indicating the existence of
stem cell population in pulp tissue originate from
the observations made on the repair process
occurring in dentin following carious lesions.
The cells responsible for reparative dentin for-
tion are thought to arise from the precursor
cells, residing somewhere within the pulp tis-
sue. In this regard, a study by Tsukamoto
and colleagues showed that fibroblastic cells
from pulp of deciduous and supernumerary teeth
were able to produce dentin like nodules at cul-
ture. Similar results were reported by Couble
et al in an explant culture of pulp tissue in a me-
dium containing glycerol phosphate.
Definitive evidences for the existence of
stem cells in pulp tissue was provided by Gron-
thos et al, who culture-expanded fibroblastic
cell population from pulp tissue, and examined
their differentiating potential both in vitro and in vivo. According to their reports, the cells
tended to differentiate into bone and neural cell
lineages in vitro, and produced dentin-
producing cells in vivo. Subsequent investi-
gations indicated the bone differentiating po-
tentials of the dental pulp stem cells as well. Zhang et al reported to succeed in
differentiating dental pulp-derived stem cells
into more cell lineages than those that had
been reported before.
According to the Stem Cell Committee of
International Society for Cell Therapy, MSCs are
cells capable of growing as adherent cells, differentiating among bone, cartilage and adi-
pose cells, and expressing some certain sur-
face markers including CD105, CD73 and
CD90, but nonhematopoietic-endothelial epi-
topes such as CD45, CD34, CD11b and CD19. The studies that have so far been car-
rried out regarding pulp stem cells have not
made it clear whether or not the fibroblastic
cells from pulp tissue, which are capable of
differentiating into odontoblast-like cells, meet
these criteria.

The objectives of the present study were
three folds: 1- to isolate and culture expand
the stem cells from dental pulps of human third
molars, and to differentiate them among odon-
toblastic cell lineages in vitro, 2- to find out
whether or not the cells capable of producing
odontoblastic cells are of MSC population de-
scribed elsewhere (i.e. the cells with differenti-
tating capacity into bone, cartilage and adipose
cell lineages and with characteristic surface
antigen profile that was mentioned above), and
3- to investigate some growth kinetics of dental
pulp-derived stem cells.

Materials and Methods

Pulp Tissue and Cell Culture
Five human third molars were used to har-
vest the pulp tissue for experimentation. The
teeth were obtained from young adult patients
(20-25 years old) at the dental clinic of Shahid
Beheshti University of Medical Sciences using
the guidelines approved by the Ethic Commit-
tee of Royan Institute. The specimens were
transferred to Adult Stem Cell Lab of the
Royan Institute for stem cell isolation. To re-
veal the pulp chamber, tooth surfaces was cut
around the root-enamel boundary using dental
fissure burs. Pulp tissue were then gently col-
lected from the chambers, and subjected to
enzymatic digestion using an enzyme solution
consisting of 3 mg/ml collagenase type I and 4
mg/ml dipase (both from Sigma, Germany) for
30 minutes at 37°C. Three ml of Dulbecco’s
Modified Eagle Medium (DMEM, Gibco, Ger-
many) were added to the digest followed by
supplementation with 15% Fetal Bovine Serum
(FBS, Gibco, Germany) and centrifugation at
1200 rpm for 5 minutes. The pellet was then
suspened in fresh proliferation medium consist-
ing of DMEM supplemented with 15% FBS,
100 IU/ml penicillin and 100 µg/ml streptomycin
( both antibiotics from Sigma, Germany) plated
in 6-well culture plates at 1000
cells/well. The plates were then incubated in
an atmosphere of 5% CO2 and a temperature
of 37°C. Three days after the culture initiation,
the medium was removed, the cells were
washed with phosphate Buffer Solution (PBS),
and fresh medium was added. The culture me-
dium was changed every three days until the
cultures confluece was achieved. Afterwards,
the cultures were trypsinized, and subcultured
at a ratio of 1:3 (passage 1). Two additional
subcultures were performed to multiply the
cells, which were then used in the following
experiments.

Flow Cytometric Analysis of Cell Surface
Epitopes
Flow cytometric analysis was used to de-
termine the surface phenotype profile of the
isolated cells. For this purpose, about 10⁶ cells
of passaged-3 cultures, 5 µl of either Propidium Iodide (PE) or Fluorescein Isothiocyanate (FITC) -conjugated antibody and 5 µl of blocking buffer were placed in 5 ml tubes and incubated at 4°C for 20−25 min in a dark place. Isotopes of Immunoglobulin G2 (IgG2) and Immunoglobulin G1 (IgG1) were used as control groups. The cells were washed with 1 ml washing buffer (PBS supplemented with 1% FBS) and centrifuged at 1200 rpm. The cell pellet was then suspended in 300−500 µl washing buffer and analyzed by flow cytometry (FACS calibur cytometer equipped with 488 nm argon lasers). WinMDI software was used to analyze the flow cytometric results. The cells were stained using FITC-conjugated CD31, CD33, CD45, CD90, CD105 and PE-conjugated CD11b, CD34, CD44, CD56, CD73 and CD19 antibodies (all from Becton Dickenson).

Odontoblast Differentiation

Stem cells derived from the pulp of human third molars, at passage 3, were cultivated at 10^5 cells in 6-well culture plates in proliferation medium and allowed to reach confluence. Afterwards, the medium was substituted with differentiating medium including DMEM supplemented with 0.5 µM vitamin D3 (Sigma, USA), 50 mg/ml ascorbic acid 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA)21 The cultures were then incubated for the next 3 weeks during which the medium was changed twice weekly. At the end of differentiation period, the cultures were analyzed for odontoblast differentiation by either alizarin red staining for mineralization or by RT-PCR for odontoblast specific gene expression.

Differentiation among Mesenchymal Cell Lineages

Osteogenic culture: The passaged-3 dental pulp cells were plated at 10^5 cells in 6-well culture plates in the proliferation medium and allowed to become confluent. The medium was then replaced by differentiating medium composed of DMEM supplemented with 50 µg/ml ascorbic acid 3-phosphate, 100 nM dexamethasone and 50 µg/ml insulin transferin selenium+ premix (Sigma, USA), 1.25 mg bovine serum albumin (Sigma, Germany), 50mg/ml insulin transferin selenium+ premix (Sigma, Germany), 10 ng/ml bone morphogenetic protein-6 (BMP6, Sigma, Germany), 50 µg/ml indomethacine.22 The cultures were incubated at 37°C and 5% CO2 for three weeks with medium changes of twice weekly. To prepare the cultures for the assessment of cartilage differentiation, the pellets were fixed with 10% formalin, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Five-µm-thick sections were then made and stained by toluidine blue. Further examination was done using RT-PCR analysis for cartilage specific gene expression in the culture.

RT-PCR Analysis

Total RNA was isolated from the differentiated cells using the RNX™ (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). In order to eliminate residual DNA, the samples of the isolated RNAs were treated with 1U/ml of RNase-free DNasel (EN0521; Fermentas,Opelstrasse 9,Germany) per 1 mg of RNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311; Fermentas, Germany) and 1×reaction buffer with MgCl2 for 30 min at 37°C. To inactivate the DNasel, 1 ml of 25mM EDTA was added to the sample which was then incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using oligo (dt) as a primer and a RevertAid™ First Strand cDNA Synthesis Kit (K1622; Fermentas, Germany) according to the manufacturer’s instructions. To provide a negative control in the subsequent PCR, for every reaction set, one RNA sample was prepared without RevertAid™M-MuLV Reverse
Transcriptase (RT reaction). To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 ml cDNA, 1×PCR buffer (AMSTM; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer (table 1), and 1U Taq DNA polymerase.

**Growth Characteristics of the Cells**

**Colony Forming Unit-Fibroblast (CFU-F) Assay:** MSCs are colonogenic cells, and CFU-F assay is routinely used to assess the potential of in vitro growth of MSCs. To determine the colonogenic ability of the pulp-derived MSCs, 100 cells of the passaged-3 culture were plated in a 25-cm²-plastic flask in the DMEM, supplemented with 15% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and were incubated at 37°C and 5% CO2 for a period of 10 days. At the end of the cultivation period the number of the colonies was determined using an inverted light microscope.

**Population doubling time (PDT):** It is defined as the time by which a given cell population doubles in number. It is indeed an indication of the rate of proliferation of the given cell population in culture. To determine PDT of the isolated cells from dental pulp, passaged-3 cells were plated at 250 cells/well of 12-well culture plate in a DMEM supplemented with 15% FBS and 100 IU/ml penicillin, 100 µg/ml streptomycin and incubated for a period of 10 days at the end of which the cell were lifted and counted with a hemocytometer. Population doubling time was determined using the following formula, where \( N_0 \) is the initiating cell number (which was 250 cells), \( N \) is the harvesting cell number (which was 512800 cells) and \( C.T \) is the culture time (which was 10 days).

\[
(PDT) = \frac{C.T \times 3.31}{\log \left(\frac{N}{N_0}\right)}
\]

**Daily examination of cells growth:** Cells in culture grow in a specific pattern consisting of lag phase (adaptation phase), log phase (proliferation phase) and plateau. To determine these features for dental pulp MSCs, passaged-3 cells were counted and plated at 250 cells per well in 12-well culture plates and allowed to achieve confluence. During the culture time, the number of cells was determined on a daily basis with a hemocytometer. The growth curve for the culture was plotted using the average values of daily cell numbers.

**Results**

**Cell Culture**

Dental pulp cells were observed to grow with colony formation at primary culture. The colonies were mainly composed of fibroblastic cells. A number of small clear cells were also observed on the fibroblastic cells (figure 1A). The cultures tended to achieve confluence in 10 days. Confluent cultures were composed of multiple bundles of fibroblastic cells each running in a particular direction, a feature typical of human marrow-derived MSC culture (figure 1B). Dental pulp-derived fibroblastic cells tended to proliferate at a faster rate than the cells in the primary culture, so that the subcultures reached confluence in about 6 days. They retained fibroblastic morphology at all subcultures.

**Flow Cytometry**

Flow cytometric results showed that CD105, CD90, CD44 and CD73 were expressed on the majority (more than 97%) of

| Table 1: The list of the primers used in RT-PCR experiments |
|----------------|----------------|----------------|--------------------|
| **Gene name** | **Direction** | **Sequence** | **Accession number** |
| DMP1           | Forward       | GCAGAGTGATGACCCAGAG | NG_008988.1       |
|                | Reverse       | GCTCGCCTTCTGATCTTCC |                  |
| DSPP           | Forward       | CCATTCCAGTTCTCTAAGCC | NG_011595.1      |
|                | Reverse       | CTGCCCACTTAGAGCCACC |                  |
| Aggrecan       | Forward       | TCAAGACACATAAGACCCAGAC | NM_001135.2     |
|                | Reverse       | AGCGAAGAGAGAGAGCACC |                  |
| Collagen type II| Forward      | GCAGGAGAGGAGGAGGAGAG | NG_008072.1     |
|                | Reverse       | AACTGTGAGGGGTGGGATG |                  |
| PPAR-Gamma     | Forward       | CTAAGAGCTGCCAGAAG | NC_000003.11 |
|                | Reverse       | TGCTCTGCTTCTGCTTCGTGAG |                |
| PPAR-Alpha     | Forward       | ACTCGGCTTCTTGGATGAT | NC_000022.10 |
|                | Reverse       | CAGGAGCGAGACCCCTAGA |                  |
| RunX2          | Forward       | CAATGAGAGGGATGCAACGA | NG_008020.1 |
|                | Reverse       | GGCGCGGCGGAGGAGGAGG | NC_000001.10 |
| Osteocalcin    | Forward       | GCAGCAGGAAAGAAGAAGAAG |             |
|                | Reverse       | CAGGAGCGAGACCCCTAGA |                  |
| GAPDH          | Forward       | CTCATTCTTCTGCTTACACC | NC_000012.11 |
|                | Reverse       | CTTCCTCTTGCTTCTTGT |                  |

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the isolated dental pulp cells, but CD56, CD11b, CD34, CD31, CD33 and CD45 were expressed in a very small percentage (less than 1-2%) of them. CD19 was expressed on about 13% of the studied cells (figure 2).

**Odontoblast Differentiation**

The dental pulp cells cultivated in odontoblast-inducing conditions were seen to undergo some morphologic reorganization including the formation of cell aggregates at some area of culture plate. This area appeared to be a foci of differentiation in which mineralized matrix deposited by the differentiating cells was positively stained with alizarin red (figure 3A). According to RT-PCR analysis the odontoblast-specific mRNAs including Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) were largely produced in differentiating cells (Figure 3B). All such evidences were indicative of successful odontoblast differentiation of the dental pulp cells in the culture conditions.

**Differentiation among Mesenchymal Cell Lineages**

In some areas of osteoinductive cultures, nodule-like structures were observed. Following the alizarin red staining, red mineralizing areas were formed (figure 4A).
Differentiation was further demonstrated by RT-PCR detection of the osteocytic markers including osteocalcin and Runx2 mRNA in the cultures (figure 4D).

The potential of adipogenic differentiation in dental pulp MSCs appeared to be relatively weak, since a few cells per microscopic field were observed to be differentiated into adipocyte in differentiation culture. The lipid droplets developed at adipogenic cultures stained red with Oil red staining (figure 4B). According to RT-PCR results, adipose related-genes such as Peroxisome proliferators activated receptor-alpha (PPAR-alpha) and Peroxisome proliferators activated receptor-gamma (PPAR-gamma) were successfully expressed in the cultures (figure 4D).

In the micro mass culture system for chondrocyte differentiation, metachromatic matrix was detected by toluidine blue staining (figure 4C). Moreover RT-PCR results indicated the expression of cartilage-specific-genes including aggrecan and collagen II by the differentiating cells (figure 4D).

Colonogenic Assay
Dental pulp MSCs were produced an average of 57.4 colonies per each 100 cells per culture flask.

Population Doubling Time
The PDT for dental pulp MSCs was about 21.89 hours.

Growth Curve
The curve plotted for dental pulp MSCs revealed that the cell population had a lag phase of about 3 days, after which they multiplied at a relatively rapid rate and reached plateau on the day 9 of culture period (figure 5).

Discussion
In the present study a population of fibroblastic cells with differentiation potential into odontoblast cells from human third molar was isolated and culture expanded as had been done previously. Moreover, the study examined whether or not the isolated cells belonged to mesenchymal stem cells described elsewhere. The findings indicate that the cells were succeeded in passing all the examinations related to MSCs including expressing some mesenchymal lineage surface epitopes, not expressing endothelial and hematopoietic markers, growing as adherent cells, and differentiating into bone, cartilage and adipose cells in vitro. All these properties are criteria that indicate the mesenchymal stem cell nature of the fibroblastic cells.
derived from human third molar. In a previous report, Zhang and colleagues demonstrated the tripotent differentiation potential of the human dental pulp stem cells, but they failed to show the surface antigenic profile of the isolated cells. Moreover, Yalvac et al studied the

Figure 4: Differentiation of human dental pulp stem cells among skeletal cell lineages. A- Alizarin red staining for osteogenic differentiation indicated the mineralized bone matrix deposition among the cells, B- Lipid droplets within the adipogenic cells stained red by Oil red staining, C- Following toluidine blue staining, methachromatic matrix (a purple color) was appeared in the section prepared from chondrogenic micro mass culture of the pulp stem cells, D- RT-PCR analysis indicated the expression of osteogenic (osteocalcin and Runx2), adipogenic (peroxisome proliferators activated receptor-alpha (PPAR alpha) and PPAR gamma) and chondrogenic (aggrecan and coll II) genes at relevant cultures.

Figure 5: A representative growth curve plotted after daily determination of the dental pulp stem cells in vitro proliferation.
Mesenchymal stem cells have so far been isolated and characterized from a multitude of sources including, cord blood, peripheral blood, amniotic fluid, cartilage, bone and adipose tissue. Each of these sources and the isolated cells from them have some advantages and disadvantages that have been mentioned in the related literature.\(^5\)\(^-\)\(^11\) Mesenchymal stem cells from dental pulp have the advantage of being derived from a tissue that is routinely discarded, if not being used. These cells are of utmost importance in regenerative medicine involving dentin. It should be noted that tooth is a complex tissue made from two cell types: odontoblast, which are responsible for dentin production, and ameloblast, which are responsible for enamel deposition. Therefore, creating a whole tooth would be a highly complex process requiring multiple cell types, but partial repair of a tooth would be much more feasible. Dentin-producing MSCs can be used for this purpose in future regenerative medicine.

The present study did suffer from a number of limitations. One limitation was related to the inherent scarcity of pulp tissue in pulp chamber of the human teeth. Due to the small volume of pulp tissue, a number of attempts to establish pulp-derived cell culture were failed. The other limitation was the teeth-induced contamination, which resulted in the contamination of cultures. The contamination could occur either during tooth extraction or transfer to the cell culture lab.

In order to characterize the human third molar pulp stem cells, a number of appropriate growth indices were examined. These include colonogenic ability, PDT and growth curve of the cells. Such indices were helpful in estimating the culture period required by the cells to multiply into adequate number. In MSCs applications, such as the use in tissue engineering or transplant, a huge number of cells are usually required. In such cases, culture expansion of the cells is inevitable, since the cells in the tissue of origin are scarce.

A study by Suchanek and colleagues\(^25\) reported that PDT for dental pulp stem cells from human third molar was 42.56 hours, while it was 21.89 hours in the present study. Such a discrepancy might have been due to the differences in FBS concentrations in the cell culture medium. Whereas in the current study, the culture medium was supplemented with 15% FBS, the culture medium in the study by Suchanek et al had 10% FBS.\(^25\) In agreement with this speculation, these authors showed that PDT was 55.43 hrs when a concentration of 2% was used.

Previous investigations indicate that the adipose differentiation of the pulp stem cells has been a subject of controversy. While Gronthos and colleagues reported that the fibroblastic cells capable of differentiating into odontoblast like cells \textit{in vivo} and neural cells \textit{in vitro} had no adipogenic potential,\(^16\) some others reported that the dental pulp cells could successfully differentiate into adipose cells.\(^19\)\(^,\)\(^24\)\(^-\)\(^26\) The findings of the present study indicate that lipid-droplet in stem cells developed at adipogenic conditions. These cells were positively stained by Oil red, and exhibited the expression of adipose specific genes. However, the adipogenic differentiation tended to be far less extensive that a few lipid-containing cells were observed in each microscopic field. Taken together, it seems that the adipogenic differentiation of pulp stem cells was not as strong as their bone or cartilage differentiation. More investigation is required to elucidate the underlying causes and mechanisms of such a disparity.

An important issue in stem cell investigations is the identification and confirmation of the nature of the cells that are isolated and culture-expanded. The best method for such a purpose would be to use specific surface markers of the cell. There is no a specific surface marker for mesenchymal stem cells, however, several non-specific surface antigens have been reported to exist for them.\(^27\)\(^-\)\(^29\) To overcome this problem, MSCs Committee of International Society for Cell Therapy has proposed a panel of surface antigens, which their existence must be verified on putative human MSCs before recognizing and presenting them as mesenchymal stem cells. According to the published guidelines of the Society, a panel of endothelial and hematopoietic cell surface markers must not be expressed on MSC surfaces, and a set of cluster of differentiation must be exhibited.\(^29\) The present study did examine such CDs to recognize and confirm the isolated cells as MSCs.

Taken together, the preset study showed that fibroblastic cells isolated from human third molars were able to differentiate among odontoblastic cell lineages. Moreover, it demonstrated that these cells were capable of producing osteocytic, chondrocytic and adipocytic cell lineages in culture. Furthermore, the study showed that the isolated stem cells did exhibit the expression of a number of mesenchymal-related surface antigens, but not the expression of endothelial-hematopoietic-
related markers. Therefore, it might be possible to conclude that the isolated stem cells belong to a family of MSCs that have previously been shown to be isolated from a multitude of tissues. Future quantitative studies such as the comparisons of MSCs from dental pulp with those from other tissues including bone marrow, adipose tissue, umbilical cord blood, amniotic fluid in terms of their differentiation potential into bone, cartilage and adipose cell lineages as well as surface marker profiles and growth kinetics in vitro will shed more light on the nature of such stem cells.

Conflict of Interest: None declared

References