Original

Characterization of SSEA-3 Positive Cells Derived from Human Dental Pulp Stem Cells

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Abstract: Mesenchymal stem cells are known to exist in pulp tissue. However, the detailed characteristics of the stem cells have not been elucidated. Recently, the presence of novel Muse cells which has been reported. Researches mentioned that Muse cells exist in all mesenchymal tissues such as in the bone marrow, skin and adipose tissue. However, there is hardly any report regarding the existence of Muse cells in oral tissues. Therefore, the purpose of the study was to determine the presence of Muse cells in dental pulp tissue. About 0.5% of SSEA (Stage-Specific Embryonic Antigen)-3 positive cells were isolated from dental pulp cells by cell sorter. The isolated cells showed self-renewal ability in addition to expressing the genes representing the three germ layers. Furthermore, the isolated cells showed a high calcification ability when subjected to calcification-inducing medium. Calcification ability was shown using alkaline phosphatase staining, alizarin red staining and quantification of calcium. The results suggest that SSEA-3 positive cells derived from human dental pulp tissue are similar to Muse cells, highly capable of differentiating into osteogenic cells. Isolated these cells from the dental pulp tissue is highly advantageous since they are less invasive compared to other cells. Hence, the dental pulp tissue is envisioned to be the source of new cells beneficial in regenerative medicine.

Key words: Dental pulp, SSEA-3, Flow cytometry

Introduction

Currently, researches on stem cells for regenerative medicine are highly advancing. Studies on ES cells^{1, 2)}, iPS cells^{3, 4)} and other cells used in regenerative medicine are making progress. In 2011, mesenchymal cells positive to both SSEA-3 and CD105, manifested their ability to differentiate into various cells of the body just like iPS cells and ES cells. These cells were called Muse cells⁵⁾. One characteristic of Muse cells is their non-tumorigenicity unlike iPS cells or other stem cells^{5, 6)}. Muse cells are transplanted via blood vessels and accumulate in injured sites. Studies mentioned that Muse cells spontaneously differentiate into cells in response to tissue repair and regeneration of damaged tissues^{5, 7)}, thus, Muse cells can be utilized in advanced regenerative medicine. Since Muse cells are mesenchymal in origin, a percentage of those cells can be found on any tissue in the body. Thus far, major studies revealed that Muse cells can be harvested from bone marrow, skin, adipose tissues^{5, 8-12)}. Notwithstanding, only a few studies showed the presence of Muse cells in oral tissues collected from the periodontal ligament¹³⁾.

Gronthos et al.¹⁴⁾ published a study on the existence of stem cells in pulp tissue. They found out that the odontoblasts that differentiated from dental pulp stem cells possess various characteristics such as self-renewal and differentiation¹⁵⁾. Subsequent studies mentioned the differentiation of dental pulp stem cells into nerve cells, adipocytes, chondrocytes, etc, as well as during bone formation¹⁶⁻²²⁾. Moreover, the use of dental pulp stem cells is not limited to Dentistry in terms of dentin and bone regeneration. In fact, its application in systemic diseases has been reported. Arthur et al suggested that it is possible for dental pulp stem

cells to differentiate into cells having neural functions, thus can be applied to treat neurological disorders²³⁾. Furthermore, Ikeda et al suggested that dental pulp stem cells have a superior proliferative capability and that damaged liver tissues can recuperate with its application²⁴⁾. As described above, dental pulp cells are very promising cells for regenerative medicine yet no report on Muse cells isolated from dental pulp tissue was found. Therefore, in this study, we aimed to isolate and characterize cells from human dental pulp stem cells (HDPSC) having similar capabilities with Muse cells.

Materials and Methods

Cells

Commercial dental pulp cells (HDPSC, Lonza, Inc., Basel, Switzerland) were used in the experiment with the approval of the ethics committee (Ethics Committee approval number: 1113).

Cell culture

Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Carlsbad, CA, USA) was the growth medium used supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Co., St. Louis, MO, USA) and 0.1 mg/ml kanamycin (Life Technologies, Inc., CA, USA). HDPSC, 3.0×10^5 , were seeded in T75 flasks and cultured in an incubator under 5%CO₂ condition. The medium was changed every 72 hours. Upon reaching subconfluence, exfoliation and passage of the cells were performed using Trypsin-EDTA (Life Technologies, Inc., Carlsbad, CA, USA). Cells were cultured until reaching over confluence and used for sorting after three passages.

Flow cytometry and cell sorting

Upon reaching over confluence at the 3rd passage, HDPSC were de-

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tached and harvested using Trypsin-EDTA. Anti-SSEA-3 (Biolegend, Inc., San Diego, CA, USA) was used as the primary antibody. Staining was performed using FITC conjugated anti-rat IgM (Jackson ImmunoResearch, Inc., West Grove, PA, USA) as the secondary antibody. EDTA-2Na of 2mM (Nacalai Tesque, Inc., Japan) was added to phosphate buffered saline (PBS, Sigma-Aldrich, Co., St. Louis, MO, USA). Sorting was performed with a flow cytometer (Sony, Co., Tokyo, Japan) using FACS buffer prepared by adding 0.5% bovine serum albumin (BSA, Nacalai Tesque, Inc., Japan).

Observation of cell growth

In order to observe the proliferation ability of SSEA-3 positive cells obtained by sorting, 1.0×10^4 cells were seeded on a 6-well plate and cultured using a growth medium. The medium was changed every 72 hours. Cell counting was carried out for 10 days immediately after sowing with a 24-hour interval.

Observation of cluster formation and self-renewal ability

Cells sorted from HDPSC were cultured in poly 2-hydroxymethyl methacrylate (Sigma-Aldrich, Co., St. Louis, MO, USA) on a 96-well plate. The medium was added once every 3 days and floating culture was performed. Clusters were identified as a group of cells having a diameter of 25 μm or more. Cluster formation from individual cells was observed for 5 to 7 days after seeding. Formed clusters were then collected and seeded on a gelatin-coated 24-well plate for cell adhesion culture. RNA was harvested from the attached, proliferated cells and those individual cells were resuspended to determine their capability of forming clusters.

The ability of the cells to self-replicate was assessed by observing whether individual cells maintained in resuspension culture would form clusters after attaching and culturing from clustered cells.

Gene expression representing the three germ layers (Quantitative PCR: Q-PCR)

Total RNA was collected using Nucleo spin RNA XS (Macherey-Nagel, GmbH & Co., KG., Duren, Germany), and reverse transcription to cDNA was performed using SuperScript VILO cDNA Synthesis Kit (Life Technologies, Inc., Carlsbad, CA, USA). PCR was carried out using FastStart Essential DNA Green Master (Roche, Ltd., Basel, Switzerland). The expression of hGAPDH (an endogenous control), α -feto-protein (AFP) and GATA6 (endodermal markers), Brachyury (a mesodermal marker) and Nestin (an ectodermal marker) were tested. Each primer design is shown on Table 1.

Calcification induction (Alkaline Phosphatase [ALP] staining, Alizarin red staining, Ca quantification)

Calcification was induced on cells sorted from adhered cells. Cell count of 3.0×10^4 was seeded on a 6-well plate, cultured in a growth medium and placed in an incubator at 37°C and under 5% CO_2 . After adhesion was confirmed the next day, calcification medium (10% FBS, 10 nmol/l dexamethasone, 0.05 $\mu\text{mol/l}$ ascorbic acid, 10 $\mu\text{mol/l}$ beta sodium glycerol phosphate added DMEM) was added and replaced every 72 hours. ALP and alizarin red stainings were performed at week 1, 2 and 3 after inducing calcification and the amount of Ca deposited was determined.

ALP staining

Cells on a 6-well plate, subjected to calcification after 1, 2 and 3 weeks were stained with ALP. After removing the culture supernatant,

Table 1. PCR primers

Gene		Primer Sequence 5'→3'
hGAPDH	Sence	:ATCAAGAAGGTGGTGAAGCA
	Antisence	:GTCGCTGTTGAAGTCAGAGGA
α -fetoprotein	Sence	:CCACTTGTGCGCAACTCAGTGA
	Antisence	:TGCAGGAGGGACATATGTTTCA
GATA6	Sence	:CCTGCGGGCTCTACGCAAGATGAAC
	Antisence	AAGGGTGCAGGAGACACAGATAC
Brachyury	Sence	:GAACAGCTCTCCAACTATG
	Antisence	:AGACTGGGATACTGGCTAGAG
nestin	Sence	:TGCGGGCTACTGAAAAGTCC
	Antisence	:TGTAGGCCCTGTTTCTCTCTG

Figure 1. Experiment flowchart

the cells were washed with PBS, fixed with 100% methanol and then washed again with PBS three times. Subsequently, the adjusted substrate solution was added to each well and allowed to react for 30 minutes at 37°C. Thereafter, the cells were washed again with PBS three times, dried and finally, the staining was assessed.

Alizarin red staining

Cells on a 6-well plate subjected to calcification after 1, 2 and 3 weeks were subjected to Alizarin red S staining. After removing the culture supernatant, the cells were washed with PBS, fixed with 100% ethanol, and then washed again with distilled water three times. Next, 1.0% alizarin red S staining solution was added and the mixture was allowed to react for 10 minutes at room temperature. Thereafter, the samples were washed again with distilled water three times, dried and the staining was assessed.

Ca quantification

The amount of calcium formed on a 6-well plate was measured at 1, 2 and 3 weeks after induction. After removing the culture supernatant, the cells were washed with PBS and adherent cells were lysed using 0.5 M hydrochloric acid. The suspension was used for measurement. Calcium E-Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was performed where samples were placed on a plate reader at a wavelength of 570 nm to calculate the amount of calcium.

Fig. 1 shows the outline of the experiment.

Figure 2. Preparation of SSEA-3 positive cells from HDPSC. About 0.5% SSEA-3 positive cells were isolated from HDPSC.

Figure 3. Cell proliferation ability of HPDSC-derived SSEA-3 positive cells. When the HDPSC-derived SSEA-3 positive cells were cultured in a 6-well plate, proliferation ability similar to typical mesenchymal cells was evident and the confluence was reached 10 days after seeding.

Results

Flow cytometry and cell sorting

HDPSC that reached over confluency were stained with anti-SSEA-3 antibody and then sorted using FITC. About 0.5% of SSEA-3 positive cells were isolated from HDPSC (Fig. 2).

Observation of cell growth potential

Fig. 3 shows the growth curve drawn for 10 days of sowing the HPDSC-derived SSEA-3 positive cells. The graph shows the increasing growth potential of the isolated cells.

Observation of cluster formation and self-renewal ability

The SSEA-3 positive cells sorted from HDPSC were floated and cultured on a poly-HEMA coated 96-well plate for each individual cell. Clusters exceeding 25 μm in diameter were examined (Fig. 4A). Clusters were adhered on gelatin-coated dishes and then detached and seeded

Figure 4. Cluster formation and observation of self-renewal ability of HDPSC-derived SSEA-3 positive cells. HDPSC-derived SSEA-3 positive cells isolated from cell sorting were floated and cultured on a poly-HEMA coated 96-well plate individually. Cells were able to form clusters greater than 25 μm in diameter(A). These clusters adhered and proliferated on gelatin-coated dishes. The clusters were separated and allowed to grow again individually in poly-HEMA coated 96-well plate after seeding(B). Scale bar: 50 μm .

Figure 5. Observation of three embryonic gene expression of HDPSC-derived SSEA-3 positive cells. For verification of pluripotency, gene expression representing the three germ layers was analyzed by quantitative-PCR. The isolated cells expressed the genes representing the three germ layers (α -fetoprotein [AFP], GATA6, Brachyury, nestin). Mean $\pm$ standard error (n=3).

Figure 7. Calcium quantification of HDPSC-derived SSEA-3 positive cell at 1, 2 and 3 weeks after calcification induction. Mean $\pm$ standard error (n=3). The amount of calcium was measured at 1, 2 and 3 weeks after initiation of calcification in HDPSC+ cultured in a 6-well plate. Calcium concentration increased over time.

once more on a poly-HEMA coated 96-well plate to determine its capability to form clusters again. Cultured individual cells again were able to form clusters indicating their self-renewal ability (Fig. 4B).

Gene expression representing the three germ layers (Q-PCR)

Verification of pluripotency was carried out by analyzing the gene expression representing the three germ layers by Q-PCR based on RNA collected from SSEA-3 positive cells sorted from HDPSC. Results showed that all genes representing the three germ layers were expressed. Particularly, Nestin, which is a gene expressed by the ectodermal cells, was strongly expressed (Fig. 5).

Calcification induction (ALP staining, Alizarin red staining, Ca quantification)

ALP staining

SSEA-3 positive cells on a 6-well plate were cultured in a calcification medium followed by staining with ALP at 1, 2 and 3 weeks after induction. Intense ALP staining was confirmed on HDPSC-derived SSEA-3 positive cells (Fig. 6A).

Alizarin red staining

SSEA-3 positive cells on a 6-well plate were cultured in a calcification medium followed by staining with alizarin red at 1, 2 and 3 weeks after induction. Intense alizarin red was observed on HDPSC-derived

(A)

1w

2w

3w

(B)

1w

2w

3w

Figure 6. ALP and alizarin red stained images at 1, 2 and 3 weeks after mineralization induction in HDPSC-derived SSEA-3 positive cells. (A) ALP staining (B) Alizarin red. Staining for both ALP and alizarin red increased over time.

SSEA-3 positive cells and the intensity progressed over time. Calcified substances were clearly stained at 3 weeks confirming its high calcification ability (Fig. 6B).

Ca quantification

We measured the amount of calcium at 1, 2 and 3 weeks after inducing calcification on SSEA-3 positive cells cultured on a 6-well plate (Fig. 7). Calcium concentration increased over time and prominent calcium production was confirmed 3 weeks after calcification induction.

Discussion

Mesenchymal stem cells are present in soft tissues in the oral cavity and it is thought that these stem cells have functional roles on periodontal tissue regeneration using materials such as GTR or enamel matrix protein. It is known that stem cells positive to STRO-1, SSEA-4 and MUC-18 are present in oral tissues but the existence of pluripotent Muse cells that are positive to SSEA-3 and CD105 is uncertain^{5, 8-12}.

The ability of Muse cells to form clusters all over again from individual cells on floating culture has been recognized⁵. In this experiment, about 0.5% SSEA-3 positive cells were sorted out from HDPSC. The proliferation ability of the isolated cells was almost similar to the SSEA-3 positive cells harvested from the periodontal ligament and skin¹³ showing the same growth curve exemplified by typical undifferentiated mesenchymal cells. In addition, cluster formation was observed when individual cells were cultured on a suspension medium. Self-renewal ability was confirmed when cells from the original clusters were separated and then were able to form clusters for the second time. Furthermore, Q-PCR was carried out to determine the gene expression representing the three embryonic layers. Endodermal markers such as AFP and GATA6, mesodermal marker Brachyury, and ectodermal marker Nestin were all expressed. Based on the results, the isolated cells are considered to be Muse cells present in HDPSC. We thought that many mesenchymal stem cells are contained in the dental pulp tissue and so

we considered that a number of Muse cells would also be isolated from HDPSC. However, current result showed a lower percentage of Muse cells isolated from HDPSC than those isolated from human periodontal ligament fibroblasts (HPdLF) and human normal skin fibroblasts (HN-HDF) reported earlier¹³. This was thought to be due to the fact that the dental pulp cells consist of a more differentiated cell population unlike the Muse cells which are more undifferentiated.

Mineralization was induced in HDPSC-derived SSEA-3 positive cells to determine the ability to produce calcium. Strong ALP and alizarin red stainings were obtained at 1, 2 and 3 weeks after induction and the intensity increased over time indicating that HDPSC-derived SSEA-3 positive cells have high calcification ability. Similar results were obtained when the amount of calcium deposition was measured. Calcification is highly possible to take place since HDPSC is originally responsible for dentin repair and regeneration. Thus differentiation into cell capable of producing hard tissues is high probable.

The application of pluripotent Muse cells in early regenerative medicine as is highly expected. Currently, Muse cells transported in the bloodstream, tend to accumulate, proliferate and differentiate to participate in repair and regeneration of damaged tissues. Whether those cells are part of the damaged tissue or sorted from similar tissues, Muse cells can effectively aid in tissue regeneration. In this study, we characterized the SSEA-3 positive cells and based on our preliminary study, most SSEA-3 positive cells are also positive to CD105¹³. By decreasing the sorting process, cell damage could have been avoided thus increasing the count of isolated undifferentiated mesenchymal cells. Teeth extracted for justifiable reasons contain organized tissues such as pulp and periodontal ligament and discarding them is considered medical waste. Currently, regenerative medicine using bone marrow cells is mainly performed. Nevertheless, new cells can be harvested from the pulp which are considered less invasive compared to bone marrow cells. Thus, dental pulp cells are effective source of new cells in the future.

If the cells to be used in regenerative medicine have sufficient phenotype of their source, then those cells can be efficiently isolated and regenerated to develop the corresponding body tissues. Establishing the right flow or process from cell sorting to transplantation is an important step for efficient regenerative medicine.

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Conflict of Interest

The authors declare no conflict of interest with the companies and organizations, etc in relation to the study.

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