Characterization of mesenchymal stem cells derived from periodontal ligament

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Accepted for publication, January 10, 2018

Abstract: It is well-known that mesenchymal stem cells responsible for periodontal tissue regeneration exist in the periodontal ligament by differentiation into fibroblasts, cementoblasts, and osteoblasts. However, the detailed characteristics of stem cells have not been clarified. Recently, the presence of a novel stem cell called Muse cells has been reported. It was mentioned that Muse cells exist in all mesenchymal tissues, and which can be found in the bone marrow, skin and adipose tissues. Nevertheless, the existence of Muse cells in oral tissues has not yet been reported. This study investigated whether Muse cells are present in the periodontal ligament. Results showed that about 0.83% of Muse cells sorted out from periodontal ligament cells showed the ability for self-renewal as well as the expression of genes present in all three germ layers. When compared to Muse cells derived from the skin for calcification ability, Muse cells derived from periodontal ligament showed higher calcification ability both at gene and tissue levels. Although both cells were considered Muse cells, cells derived from the periodontal ligament have different characteristics compared to the Muse cells derived from the skin. The results suggest that stem cells derived from somatic cells are possible to maintaining the original phenotypes. Therefore, when using stem cells for regenerative medicine, we suggest that the stem cells should be harvested from the same tissue, which expects repair or regeneration of the tissue.

Key words: Dermal fibroblast, Mesenchymal stem cell, Muse cell, Periodontal ligament, SSEA-3

Introduction

Currently, researchers on regenerative medicine using stem cells are actively ongoing. The study on cells used in regenerative medicine, such as ES cells, iPS cells, etc are in progress. In 2011, the presence of mesenchymal cells that are both positive to SSEA-3 and CD105 was reported showing the ability of the cells to differentiate into other cell types of the body just like the iPS cells and ES cells. Those cells were then called Muse cells. Characteristics wise, Muse cells do not have the tumorigenic property that iPS cells have.

Furthermore, Muse cells, which are accumulate in the damaged site by transplantation through the blood vessels, and spontaneously differentiate into cells corresponding to the damaged tissues, and then repair and regenerate the damaged tissue. A considerable percentage of Muse cells exist in mesenchymal cells of the body. So far, major studies cited that Muse cells can be harvested from the bone marrow, skin and adipose tissues. Nevertheless, research in the dental field has not yet been conducted and the presence of Muse cells in oral tissues has not been accepted.

The periodontal ligament membrane plays a central role in the maintenance and regeneration of periodontal tissues. This function can be maintained due to the ability of periodontal ligament cells to differentiate into fibroblasts, osteoblasts or cementoblasts depending on the situation. Periodontal ligament cells do not consist of a single cell population rather, consisting of a group of different cells such as fibroblasts, osteoblasts, cementoblasts, osteoclasts, endothelial cells, epithelial rest of Malassez, neurons and undifferentiated mesenchymal cells.

Among those cells, the undifferentiated mesenchymal cells are thought to be responsible for the pluripotency of the periodontal ligament but the definite type and the characteristics of undifferentiated mesenchymal cells in the periodontal ligament has not be elucidated. Previous studies described that cells positive to stem cell markers such as STRO-1, SSEA-4, MUC18 are present in the periodontal ligament tissue, hence, there is no doubt that stem cells are present in the periodontal tissues. In this study, we aimed to investigate the presence of Muse cells in the periodontal tissues.

Applied clinical research in Muse cells reported that when Muse cells are transplanted through blood vessels, they accumulate in damaged tissues and then differentiate and proliferate to repair and regenerate damaged tissues. Muse cells, which are isolated from somatic stem cells, are possible to retain the phenotype depending on the cell where it was harvested from. Moreover, we analyzed the characteristics of Muse cells obtained from skin and those obtained from the periodontal ligament, and examined if there is a difference in the characteristics between the same SSEA-3 and CD105 positive cells coming from two different sources.

Materials and Methods

Cells

This experiment was carried out using commercial-base human periodontal ligament fibroblasts (HPDLF, Lonza, Inc., Basel, Switzerland) and human normal skin fibroblasts (HNHDF, Lonza, Inc., Basel, Switzerland).

Cell Culture

Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Co., St. Louis, MO, USA) with 0.1 mg/ml Kanamycin (Life Technologies, Inc., Carlsbad, CA, USA) was used as the growth medium. Cell count of 3.0 x 10^5 HPDLF and HNHDF were seeded in T75 flasks respectively and cultured under 5% CO2 condition. The medium was changed every 72 hours and when the cells reached subconfluent, they were detached and passaged using Trypsin-EDTA.
Calcification was induced using cells obtained from sorting
Flow cytometry and cell sorting
At the third passage of HPDLF and HNHDF, upon reaching over
Calcification-related gene expressions (Q-PCR)
Total RNA was collected from Nucleo spin RNA XS, cultured in a growth medium. The medium was changed every 72
Calcification induction (Q-PCR, Alkaline Phosphatase staining, Alizarin Red staining, Ca determination)
Calcification was induced using cells obtained from sorting
Calcification-related gene expression at day 1, 3 and 5
Calcification-related gene expression at day 1, 2 and 3 weeks after calcification induction.
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calcium deposition was also analyzed by ALP and Alizarin red staining at 1, 2 and 3 weeks after calcification induction.
Calcification-related gene expression at day 1, 2 and 3 weeks after calcification induction.
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calcium deposition was also analyzed by ALP and Alizarin red staining at 1, 2 and 3 weeks after calcification induction.
Calcification-related gene expression at day 1, 2 and 3 weeks after calcification induction.
Flow Cytometry and Cell Sorting

After cells were sorted out upon reaching over confluence and stained with anti-SSEA-3, FITC and anti-CD105, an SSEA-3 positive rate of about 0.98% for HPDLF and about 2.0% for HNHDF were obtained (Fig. 1A-D). The CD105 positive rate was about 98.5% for both HPDLF and HNHDF cells (Fig. 1C, D). About 0.83% in HPDLF and about 1.8% in HNHDF showed double positive staining to SSEA-3 and CD105.

Results

at 1, 2 and 3 weeks after initiation of mineralization. The culture supernatant in the wells was removed, washed with PBS, and adherent cells were lysed with 0.5 M hydrochloric acid. The suspension was used for measuring the amount of calcium. Calcium E-Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used and a wavelength of 570 nm was measured with a plate reader to calculate the amount of calcium.
Observation of Cell Growth Potential

When HPDLF-derived SSEA3/CD105 positive cells and HNHDF-derived SSEA3/CD105 positive cells were cultured in a 6-well plate, both cells displayed similar growth curve and reached confluence at day 9 after seeding (Fig. 2A, B).

Observation of cluster formation and self-renewal ability

SSEA-3/CD105 positive cells obtained by sorting were floated and cultured on a poly-HEMA coated 96-well plate for each cell and then, clusters exceeding 25 µm in diameter were observed (Fig. 3A, B). These clusters adhered and proliferated by seeding on gelatin-coated dishes, cloned again by separating and then each cell was allowed to grow in poly-HEMA coated 96-well plate after seeding (Fig. 3C).

Gene expression corresponding to the three germ layers (Q-PCR)

For verification of pluripotency, gene expression corresponding to the three germ layers was observed by Q-PCR based on RNA collected from sorted SSEA-3/CD105 positive cells. Results showed the expression of genes corresponding to the three germ layers (α-fetoprotein, GATA6, Brachyury, nestin) in both cells. Nestin, an ectodermal gene, was strongly expressed in HPDLF-derived SSEA-3/CD105 positive cells (Fig. 4).

Calcification Induction (Q-PCR, Alkaline Phosphatase staining, Alizarin Red staining, Ca quantification)

Calcification related gene expression (Q-PCR)

Each SSEA-3/CD105 positive cells were cultured in a calcium-inducing medium on a 6-well plate and cDNA was obtained from total RNA extracted at 1, 3 and 5 days after induction and various
calcification related gene expressions (ALP, Runx2, COL1, OCN) were analyzed (Fig. 5). Although ALP expression increased in both cells over time, the expression in HPDLF-derived SSEA-3/CD105 positive cells is higher compared to HNHDF-derived SSEA-3/CD105 positive cells at any time. Runx2 expression decreased in HPDLF-derived SSEA-3/CD105 positive cells over time but increased in HNHDF-derived SSEA-3/CD105 positive cells. Moreover, the Runx2 expression is significantly higher in HNHDF-derived SSEA-3/CD105 positive cells compared to HPDLF-derived SSEA-3/CD105 positive cells except at day 1. COL1 expression transiently increased at day 3 then decreased at day 5 in HPDLF-derived SSEA-3/CD105 positive cells, whereas the expression in HNHDF-derived SSEA-3/CD105 positive cells continue to rise over time. A significant difference in gene expression between the two cells was observed only at day 1. Although OCN expression increased in HPDLF-derived SSEA-3/CD105 positive cells over time, there was almost no change in HNHDF-derived SSEA-3/CD105 positive cells. Moreover, OCN expressions at day 3 and 5 in HPDLF-derived SSEA-3/CD105 positive cells were significantly higher compared to HNHDF-derived SSEA-3/CD105 positive cells.

**ALP staining**

Each SSEA-3/CD105 positive cells were cultured in a calcium-
It is known that one of the characteristics of Muse cells is to form clusters from a single floating culture, and after plate culture the clustered cells, the cells form clusters again from a single floating culture. In this experiment, HPDLF and HNHDF cells which are heterogeneous cell population and more with variety cell population. Consequently, we compared the calcification ability with Muse cells derived from HPDLF and HNHDF. The expressions of ALP, Runx2, COL1, and OCN as calcification related genes were examined after culturing the cells in calcification induction medium. In HPDLF-derived cells, the ALP and OCN gene expressions increased from day 1 to day 5 after induction. Runx2 expression decreased over time from day 1 to day 5 whereas COL1 expression showed transient increase at day 3 after induction. In HNHDF-derived Muse cells, ALP expression somewhat increased over time, Runx2 and COL1 expressions showed a tremendous increase over time. The expression level of OCN did not observed the change. It seems that ALP expression which is an early calcification marker was rise up, and mineralization in HPDLF-derived Muse cells started immediately after induction of calcification. Similarly, Runx2 is an early calcification marker and the peak of its expression was noted on day 1 of induction and the expression decreased as calcification progressed. COL1 transiently increased in the initial stage of calcification and then decreased as calcification progressed. The expression of OCN increases in proportion to the calcification of cells and the amount of expression significantly increased in HPDLF-derived Muse cells. On the contrary, in HNHDF-derived Muse cells except for Runx2, gene expressions were significantly lower in ALP, COL1, and OCN than in HPDLF-derived Muse cells. HNHDF-derived cells only have higher expression in terms of Runx2 than HPDLF-derived Muse cells. This suggests that HNHDF-derived cells are slower in response to calcification than HPDLF-derived Muse cells. With ALP and Alizarin Red stainings at 1, 2 and 3 weeks of induction, HPDLF-derived Muse cells were stained darker than HNHDF-derived Muse cells at any time. It can be inferred that HPDLF-derived Muse cells have higher calcification ability than HNHDF-derived Muse cells. Moreover, when the amount of calcium deposits were measured, HPDLF-derived Muse cells had more calcium deposits than HNHDF-derived Muse cells. These results show that cells derived from HPDLF have high calcification ability with remarkable properties of periodontal ligament cells at both gene and tissue levels. HPDLF was originally responsible for the regeneration of calcified tissue and thought to be affecting periodontal tissue regeneration including bone and cementum, HNHDF present in tissues does not cause mineralization. The results suggest that even the pluripotential stem cell called Muse cells maintains the its phenotype based on its cell origin. In 2008, Aoi et al compared the properties of iPS cells established from hepatocytes, gastric epithelial cells, tail fibroblasts collected from mice. They mentioned a difference in gene expression and tumorigenicity after transplantation even if the cells were the same iPS cells depending on the harvested tissues. This result seems to support our findings.
The application of Muse cells positive to both SSEA-3 and CD105 in early regenerative medicine as pluripotent stem cells are expected. Currently, it is thought that Muse cells accumulate in damaged tissues by differentiation and then proliferate to repair and regenerate tissues transplanted via the bloodstream. However, fractionated Muse cells regenerate more effectively in the same tissue. In this regard, if Muse cells leave its phenotype of original cell source, cell sorting should be considered prior to transplantation for a more efficient tissue regeneration.

Acknowledgements

We would like to thank Shinji Shimoda and Yasuo Yamakoshi whose comments and suggestions were of inestimable value for our study.

Conflict of Interest

The authors declare no conflict of interest.

Reference
