Three-dimensional Sphere-forming Cells Are Unique Multipotent Cell Population in Dental Pulp Cells

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Abstract

Introduction: Mesenchymal stem cells (MSCs) are typically cultured as adherent monolayer using a conventional tissue culture technique. However, this technique incompletely reproduces an *in vivo* microenvironment of stem cells and results in the loss of stemness properties. Three-dimensional (3D) sphere culture is one of the most widely used 3D culture techniques that have been developed to recapitulate the *in vivo* microenvironment. However, the stemness and multilineage differentiation capacity of spheres derived from dental pulp stem cells (DPSCs) have not been well investigated. **Methods:** DPSCs were cultured and examined for the sphere-forming ability in serum-free, nonadherent conditions. The expression of pluripotency transcription factors was assayed by reverse transcription quantitative polymerase chain reaction and Western blot analysis. The expression of MSC-associated markers was determined by flow cytometry. Multilineage differentiation capacity was examined by alkaline phosphatase, alizarin red S, and oil red O assays. Subcutaneous transplantation in nude mice was used to examine the *in vivo* mineralized tissue-forming ability of sphere and adherent monolayer cells derived from DPSCs. **Results:** We showed that DPSCs form spheres. DPSC spheres exhibited a distinct stem cell phenotype characterized by robust expression of pluripotency transcription factors and decreased expression of MSC-associated markers compared with their corresponding adherent monolayer cells. Functionally, DPSC spheres exhibited enhanced *in vitro* multilineage differentiation capacity. The expression of multilineage differentiation-related genes was also highly increased in DPSC spheres. Furthermore, DPSC sphere cells possessed higher *in vivo* mineralized tissue-forming ability than adherent monolayer cells. **Conclusions:** Our findings indicate that sphere-forming cells are unique multipotent cell populations in DPSCs. Our study further suggests that DPSC spheres may provide a unique opportunity for pulp tissue regeneration. *(J Endod 2017;■:1–7)*

Key Words

Dental pulp stem cells, multilineage differentiation, sphere, stemness

Significance

Three-dimensional sphere-forming cells are unique multipotent cell populations in dental pulp stem cells. Our study suggests that these sphere-forming cell populations may provide a unique opportunity for pulp tissue regeneration.

Mesenchymal stem cells (MSCs) derived from various human tissues are heterogeneous populations of stromal cells and are commonly cultured as adherent monolayer cells using a conventional tissue culture technique. This conventional tissue culture technique incompletely reproduces the *in vivo* microenvironment of stem cells and alters the normal physiological behavior of stem cells. Thus, culturing human MSCs in the adherent monolayer resulted in the loss of stemness and multilineage differentiation capacity over time (1, 2).

To recapitulate the *in vivo* microenvironment more accurately *in vitro*, various 3-dimensional (3D) culture systems have been developed (3, 4). The formation of a 3D sphere, a spherical cluster of cells formed by self-renewal capacity in a defined condition medium, is one of the most widely used techniques for the 3D culture. Indeed, many studies have revealed differences between adherent monolayer cells and 3D sphere cells of human MSCs derived from bone marrow, adipose, and umbilical cord blood (5–8). In general, MSC spheres possess a greater multilineage differentiation capacity compared with their corresponding monolayer cells, suggesting that 3D sphere culture may better reflect the *in vivo* microenvironment of stem cells and maintain higher stemness properties (5–8). Moreover, it also suggests that the use of the 3D sphere culture technique to enrich stem cell populations is technically superior to that of the monolayer culture technique.

Dental pulp stem cells (DPSCs) are originated from neural crest ectomesenchyme (9) and express several surface markers detected on neuronal cells, all of which are not expressed MSCs from other sources (10). These results suggested that the 3D neurosphere culture technique could be used to isolate and enrich the stemlike cell population from heterogeneous DPSCs. A 3D sphere is indicative of stem cell content and

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self-renewal capacity in a given culture of heterogeneous cell populations (11). A recent study showed that DPSCs form 3D spheres in nonadherent, neurosphere culture conditions (10). However, stem cell phenotype and multilineage differentiation capacity of DPSC spheres in comparison with their corresponding adherent monolayer culture have not been well investigated.

In this study, we examined stemness phenotype and multilineage differentiation potential of DPSC spheres formed under neurosphere culture conditions in comparison with those of their corresponding adherent monolayer culture *in vitro* and *in vivo*.

Materials and Methods

DPSC Cultures and Their 3D Sphere Formation in Neurosphere Culture Conditions

Primary DPSCs were isolated from extracted human teeth and cultured in basal medium containing 10% fetal bovine serum (FBS) in alpha-minimum essential medium (Life Technologies, Grand Island, NY). To determine whether DPSCs can form spheres, we used the quantitative 3D sphere formation assay using neurosphere culture conditions. Single cells (3000, 6000, and 15,000) dissociated from the adherent monolayer culture of DPSCs were plated in Corning Costar Ultra-Low Attachment 6-well plates (Fisher Scientific, Waltham, MA) in neurosphere medium containing serum-free Dulbecco modified Eagle/F12 media supplemented with 1:50 B27 (Invitrogen, Carlsbad, CA), 20 ng/mL epidermal growth factor, 20 ng/mL, 10 μg/mL insulin, penicillin, streptomycin, and amphotericin B. The number of spheres formed were observed and counted under a microscope on day 7. Data were means $\pm$ standard deviation of triplicate experiments.

Flow Cytometry Analysis for MSC-associated Markers

After trypsinization, single cells (3000–15,000) dissociated from the monolayer culture and spheres derived from DPSCs were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes at 4°C. The cells were suspended in 2% FBS for a density of 1×10^6 cells/mL for 10 minutes at 4°C. Monoclonal antibodies against human CD146, CD271, SSEA-4, and Stro-1 (R&D System, Minneapolis, MN) or isotype-matched control immunoglobulin Gs were added to the cell suspension and incubated at 4°C in the dark for 1 hour. The labeled cells were resuspended in PBS containing 3% FBS after washing with the incubation buffer and then analyzed using a BD FACScan flow cytometer (BD Biosciences, San Jose, CA). The fluorophore-conjugated antibodies were obtained from BD Biosciences (12). This assay is quantitative assessment.

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated, and complementary DNA was made as described previously (13). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed in triplicates for each sample with LC480 SYBR Green I master (Roche, Indianapolis, IN) using universal cycling conditions on the LightCycler 480 (Roche). A total of 45 cycles were executed, and the second derivative quantification cycle value determination method was used to compare fold differences. The primer sequences are available upon request. Data are means $\pm$ standard deviation of triplicate experiments. This assay is quantitative assessment.

Western Blotting

Whole-cell extracts were isolated from an adherent monolayer culture and spheres, fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and transferred to the Immobilon membrane (Millipore, Billerica, MA). Antibodies against Oct4 (ab19857; Abcam, Boston, MA), Nanog (AF1997, R&D System), and glyceraldehyde 3-phosphate dehydrogenase (FL-335; Santa Cruz Biotechnology, Santa Cruz, CA) were used. The chemiluminescence signal was detected using the HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific, South Plainfield, NJ). The detailed protocol can be found in our previous publication (14). This assay is quantitative assessment.

Induction of Odontogenic Differentiation

Adherent monolayer or sphere cells were placed at 1.5×10^5 cells/well in a 6-well plate. When the cultures reached confluence, odontogenic differentiation was induced by incubating them in odontogenic induction medium (OIM). OIM contains alpha-minimum essential medium supplemented with 15% FBS, 100 μmol/L L-ascorbic acid 2-phosphate, 10 mmol/L beta-glycerophosphate, 10 nmol/L dexamethasone, 0.18 mmol/L KH_2PO_4, and 5 μg/mL gentamicin sulfate. After 7 days of induction, the cells were stained for alkaline phosphatase (ALP) activity *in situ* using an ALP staining kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. To determine the formation of mineralized nodules *in vitro*, cells were cultured to confluence and exposed to OIM for up to 3 weeks. The cultures were fixed in 70% ethanol for 1 hour at 4°C, rinsed in 1× PBS, and stained for 10 minutes with 40 mmol/L alizarin red S (ARS) solution at room temperature. The cells were then rinsed 5 times in H_2O followed by a 15-minute wash in 1× PBS to eliminate nonspecific staining. For the quantification of ARS staining, stained cells were destained in 10% cetylpyridinium chloride (Sigma-Aldrich) and measured at 562 nm using the microplate reader. This assay is quantitative assessment.

Induction of Adipogenic Differentiation

Adherent monolayer or sphere cells were placed at 1×10^5 cells/well in a 12-well plate. When the cultures reached confluence, adipogenic differentiation was induced by incubating them in adipogenic induction medium. Adipogenic induction medium contains Dulbecco modified Eagle medium supplemented with 10% FBS, 60 μmol/L indomethacin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 μmol/L dexamethasone, 5 μg/mL insulin, and 5 μg/mL gentamicin sulfate. After 3 weeks of induction, cells were washed once with PBS and fixed in 4% formaldehyde for 15 minutes at room temperature. Fixed cells were washed in 60% isopropyl alcohol, and lipid droplets were stained using the oil red O (ORO) staining kit (Sigma-Aldrich). After 3 consecutive washes in deionized water, the stained cells were photographed using bright-field microscopy. For the quantification of ORO staining, supernatant solutions from the stained cells were collected and measured at 540 nm using the microplate reader. This assay is quantitative assessment.

Subcutaneous Transplantation in Nude Mice

To examine whether DPSC sphere cells have enhanced odontogenic differentiation and mineralization potential *in vivo*, ectopic mineralized tissue formation was performed using nude mice as shown in our previous publication (15). Subcutaneous transplantation of the cell mixture was performed according to guidelines approved by the Chancellor's Animal Research Committee (ARC# 2006-017-01). Briefly, approximately 2.0×10^6 cells derived from DPSC spheres or their corresponding adherent monolayer culture in 40 μL medium were mixed with 40 mg hydroxyapatite/tricalcium phosphate ceramic particles (Zimmer, Carlsbad, CA). The mixture was incubated at 37°C for 2 hours with gentle shaking and then transplanted subcutaneously into the dorsal suprascapular