The use of granulocyte-colony stimulating factor induced mobilization for isolation of dental pulp stem cells with high regenerative potential

Masashi Murakami a,1, Hiroshi Horibe a,b,1, Koichiro Iohara a, Yuki Hayashi a,c, Yohei Osako a, Yoshifumi Takei d, Kazuhiko Nakata e, Noboru Motoyama f, Kenichi Kurita b, Misako Nakashima a,*

a Department of Dental Regenerative Medicine, Center of Advanced Medicine for Dental and Oral Diseases, National Center for Geriatrics and Gerontology, Research Institute, 35 Gengo, Morioka, Obu, Aichi 474-8511, Japan
b Department of Oral and Maxillofacial Surgery, School of Dentistry, Aichi Gakuin University, Nagoya, Japan
c Department of Pediatric Dentistry, School of Dentistry, Aichi Gakuin University, Nagoya, Japan
d Department of Biochemistry and Division of Disease Models, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan
e Department of Endodontics, School of Dentistry, Aichi Gakuin University, Nagoya, Japan
f Department of Cognitive Brain Science, National Center for Geriatrics and Gerontology, Research Institute, Obu, Japan

Abstract

Human dental pulp stem cells (DPSCs) contain subsets of progenitor/stem cells with high angiogenic, neurogenic and regenerative potential useful for cell therapy. It is essential to develop a safe and efficacious method to isolate the clinical-grade DPSCs subsets from a small amount of pulp tissue without using conventional flow cytometry. Thus, a method for isolation of DPSCs subsets based on their migratory response to optimized concentration of 100 ng/ml of granulocyte-colony stimulating factor (G-CSF) was determined in this study. The DPSCs mobilized by G-CSF (MDPSCs) were enriched for CD105, C-X-C chemokine receptor type 4 (CXCR-4) and G-CSF receptor (G-CSFR) positive cells, demonstrating stem cell properties including high proliferation rate and stability. The absence of abnormalities/aberrations in karyotype and lack of tumor formation after transplantation in an immunodeficient mouse were demonstrated. The conditioned medium of MDPSCs exhibited anti-apoptotic activity, enhanced migration and immunomodulatory properties. Furthermore, transplantation of MDPSCs accelerated vasculogenesis in an ischemic hindlimb model and augmented regenerated pulp tissue in an ectopic tooth root model compared to that of colony-derived DPSCs, indicating higher regenerative potential of MDPSCs. In conclusion, this isolation method for DPSCs subsets is safe and efficacious, having utility for potential clinical applications to autologous cell transplantation.

ARTICLE INFO

Article history:
Received 8 July 2013
Accepted 3 August 2013
Available online 27 August 2013

Keywords:
Dental pulp stem/progenitor cells
Granulocyte-colony stimulating factor (G-CSF)
Trophic effect
Migration
Angiogenesis/vasculogenesis
Pulp regeneration

1. Introduction

Dental pulp stem cells have many advantages for clinical applications in dentin/pulp regeneration compared with other mesenchymal stem cells (MSCs) derived from bone marrow and adipose tissue [1]. The dental pulp stem cells are easily isolated from discarded teeth following extraction with very low morbidity and no ethical issues. A higher immunosuppressivity of T-cell alloreactivity has been demonstrated in dental pulp stem cells than in bone marrow stem cells [2]. Some subfractions of adult dental pulp stem cells, such as the CD31+ side population (SP), have greater migration and higher expression of many angiogenic/neurotrophic factors than bone marrow and adipose tissue-derived stem cells of the same individual, leading to enhanced pulp regeneration [1]. On the other hand, pulp CD31+ SP cells and CD105+ cells have a greater effect on angiogenesis/vasculogenesis and neurogenesis after transplantation both in mouse hindlimb ischemia and rat brain ischemia compared with colony-derived dental pulp stem cells (DPSCs) [3,4]. Regenerated pulp
tissue is also much higher in volume and less likely to mineralize after autologous transplantation of CD31+ SP cells or CD105+ cells into the pulputomized root canal in dog teeth compared with DPSCs [4,5]. Thus, certain DPSCs subsets might be useful for cell-based therapy for angiogenesis/vasculogenesis and re-innervation/neurogenesis in ischemic diseases, nerve injury, and pulpitis [3,6–11]. Clinical trials for regeneration are currently under consideration to harness the therapeutic potential of human DPSCs subsets. However, a remaining challenge for manufacturing clinical grade human DPSCs subsets is the requirement for good manufacturing practice (GMP) grade cell isolation and processing. The safety of these CD31+ SP cells and CD105+ cells isolated by flow cytometry has not been established. On the other hand, another isolation method using immune-magnetic beads of CD34, such as the IsolX 300i Magnetic Cell Selection System device (Baxter) presently used for clinical applications [12,13], is not suitable for human DPSCs subsets because a limited volume of pulp tissue or a limited number of the primary pulp cells is available and CD34 is not a validated cell surface marker for pulp stem cell isolation. The costs will be prohibitive if CD105-magnetic beads are specially made to order for pulp stem cell isolation. Recently, methods have been further developed to isolate the MSCs in a closed system, including the Bone Marrow MSC Separation Device [14] for bone marrow and the Celution system [15] for adipose tissue. However, there are no methods or systems in place to isolate DPSC subsets with high regenerative potential for clinical use. Therefore, a cost-effective method for the isolation of DPSC subsets must be developed, leading to safe and efficacious isolation from small amounts of pulp tissue.

Hematopoietic stem/progenitor cells from the bone marrow, such as CXCR4+ cells, can be mobilized by their chemotactic reactivity in a SDF-1 gradient [16–18]. CXCR4+ dental pulp cells are localized to the peri-vascular area [19], a functional niche for stem cells. The ligand of CXCR4, SDF-1, induces a strong chemotactic response in DPSCs [1], CXCR4 is more highly expressed in fractionated pulp CD105+ stem/progenitor cells compared with colony-derived DPSCs [5] and is thought to be one of the stem cell markers. However, SDF-1 induced migration for the isolation of subsets of DPSCs cannot be used as SDF-1 has not been approved for clinical use. A potential alternative is granulocyte-colony stimulating factor (G-CSF) which is already approved by the FDA for clinical use. Many reports have revealed that G-CSF has the ability to mobilize not only hematopoietic stem cells but also MSCs from bone marrow [20,21]. Our preliminary data revealed that the migration of pulp CD105+ stem/progenitor cells toward G-CSF was significantly higher than that of DPSCs. Thus, we have devised an isolation method for a subset of DPSCs employing an optimized G-CSF-induced mobilization. The regenerative potential of dental pulp stem cells mobilized by G-CSF (MDPSCs) was compared with DPSCs in an ischemic hindlimb model and an ectopic tooth root transplantation model, and evaluated utility for potential clinical applications to autologous cell transplantation.

2. Materials and methods

This study was approved by the ethics committees and the animal care and use committees of the National Center for Geriatrics and Gerontology, Research Institute and Aichi Gakuin University. All experiments were conducted using the strict guidelines of the DNA Safety Programs.

2.1. Cell isolation and the culture conditions

Normal human third molars were collected from adults (18–29 years of age, n = 12) at the Aichi Gakuin University Dental Hospital under approved guidelines set by the School of Dentistry, Aichi Gakuin University and the National Center for Geriatrics and Gerontology, Research Institute. Dental pulp cells were isolated from human dental pulp tissue enzymatically with a slight modification of a previously described method [22]. Briefly, dental pulp tissue was minced into pieces and then enzymatically digested. The number of isolated cells and their viability was determined by trypan blue staining of the cells. The isolated cells were plated at 3 × 10⁴ cells on 35 mm dishes (BD Biosciences, Franklin Lakes, NJ) in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% human serum collected from healthy consenting adult donors. The colony-derived dental pulp stem cells (DPSCs) were detached by incubation with TrypLE Select (Invitrogen, Carlsbad, CA, USA) at 37 °C for 10 min. CD105+ cells were isolated from human dental pulp cells as described previously [23]. A human minicircle of iP-C cell line was purchased from System Biosciences (Mountain View, CA) and cultured in Repro FZ (ReproCell, Yokohama, Japan) supplemented with 5 ng/ml basic fibroblast growth factor (bFGF, PeproTech, London, UK). For feeder-free culture of iP cells, the plate was coated with 1 µg/ml Laminin-5 (ReproCell) at 4 °C overnight. NIH3T3 cells (clone 5611) were purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank (Tokyo, Japan).

2.2. Isolation of DPSC subsets by G-CSF-induced mobilization

We used a method for isolating subsets of DPSCs utilizing G-CSF (NEUROTROGIN®, Chugai Pharmaceutical Co., Ltd. Tokyo, Japan)-induced stem cell mobilization yielding the MDPSCs. Costar Transwell® (a permeable support with an 8 µm polycarbonate membrane 6.5 mm Insert, Corning, Lowell, MA), was used as the upper chamber, which was inserted into 24-well tissue culture plates, used as the lower chamber. The membrane was chemically treated using the same method in TORAYLIGHT® NV, a polysulfone membrane artificial kidney (generic name: hollow-membrane dialyzer; authorization number: 2220082X00871000) (Toray Industries, Inc., Tokyo, Japan) to prevent cell attachment. Human colony-derived dental pulp stem cells (DPSCs) (2 × 10⁴ cells/100 µl DMEM) at the second passage of culture were added to the upper chambers, and 390 µl of DMEM supplemented with 1% human serum and G-CSF was added to the lower chambers. After incubation, the medium was changed with DMEM supplemented with 10% human serum and without G-CSF.

The optimal conditions for isolation of MDPSCs were determined by varying three parameters: the cell number, the incubation time, and the G-CSF gradient. To determine the optimal cell number, 2 × 10⁴, 5 × 10⁴, and 1 × 10⁵ cells were seeded into the upper chamber: the final concentration of G-CSF (100 ng/ml) in the lower chamber and the incubation time (48 h) were fixed. After 48 h of incubation, the cells that had transmigrated were enumerated. To determine the optimal incubation time, after 12, 24, 48, and 72 h of incubation, the cells that had transmigrated were determined. The optimal cell number, 2 × 10⁴ cells, that were seeded into the upper chamber and the final concentration of G-CSF (100 ng/ml) in the lower chamber were fixed. To determine the suitable concentration of G-CSF in the lower chamber, we prepared DMEM supplemented with 10% human serum and without G-CSF. Once the cells reached 60–70% confluency, they were detached by incubation with TrypLE Select and subcultured. To evaluate the colony forming efficiency of MDPSCs, 5 × 10⁵ cells/ml of MDPSCs were seeded at the third cell culture passage on 6 cm dishes in DMEM supplemented with 10% human serum. After 4 days of culture, aggregates of ≥ 10 cells were scored as colonies.

2.3. Flow cytometric analysis

Dental pulp stem cells mobilized by G-CSF (MDPSCs) (G-CSF gradient 0, 10, 100, and 500 ng/ml), were characterized at the 6th cell culture passage, in comparison with pulp CD105+ cells, and colony-derived DPSCs (DPSCs) were used as positive and negative controls, respectively. They were immunolabeled for 60 min at 4 °C with an anti-hamster IgG negative control (Phycoerythrin-Cy7, PE-Cy7) (eBio959Arm) (eBioScience, San Diego), a rat IgG2b negative control (PE-Cy7) (RTK4330) (BioLegend), a mouse IgG1 negative control (PE) (MCA928PE) (AbD Serotec), a mouse IgG1 negative control (Allophycocyanin [APC]/[MCP-21]) (BioLegend), a mouse IgG1 negative control (Alexa Fluor 647) (MCA928A647) (BioLegend), a mouse IgG2a negative control (Fluorescein isothiocyanate [FITC] (sc-2856) (Santa Cruz), a mouse IgG2b negative control (PE-Cy3) (MPC-11) (BioLegend), and the antibodies against CD29 (PE-Cy7) (eBioHmb1-1) (eBioScience), CD31 (PE) (WM59) (BD Biosciences), CD44 (PE-Cy7) (IM7) (eBioScience), CD73 (APC) (AD2) (BioLegend), CD90 (Alexa Fluor 647) (FL5-42-1) (AbD Serotec), CD105 (FITC) (MEM-229) (Abcam, Cambridge, UK), CD146 (Alexa Fluor 647) (OT9c6) (AbD Serotec), CXC4 (FITC) (12G5) (RBDS Systems, Inc., Minneapolis), G-CSFR (FITC) (38660) (RBDS Systems), CD40 (APC) (5C1) (BioLegend), CD45 (APC) (H103) (BioLegend), CD80 (FITC) (2D10.4) (eBioScience), CD86 (PE-Cy5) (IT2.2) (eBioScience), HMC class I (HLA-A, B, and C) (PE) (3F10) (Ance1, Bayport), and HMC class II (HLA-DP, DQ, and DR) (APC) (TPR31.1) (Ance1).

2.4. Induced differentiation

The differentiation of dental pulp stem cells mobilized by G-CSF (MDPSCs) (G-CSF gradient 0, 10, 100, and 500 ng/ml) into the angiogenic, neurogenic, and odontogenic/stemogenic lineages, was determined and compared with pulp CD105+ cells and colony-derived DPSCs as described previously [24].

2.5. Proliferation and migration assays

To determine the proliferative activity in response to human serum, dental pulp stem cells mobilized by G-CSF (MDPSCs) (G-CSF gradient 0, 10, 100, and 500 ng/ml)
were compared with pulp CD105<sup>+</sup> cells and colony-derived DPSCs at the 6th passage of culture at 1 × 10<sup>4</sup> cells per well in a 96-well plate in DMEM (Sigma–Aldrich) supplemented with 10% human serum. Ten microliters of Tetra-color one<sup>®</sup> (Seikagaku Kogyo, Co., Ltd., Tokyo, Japan) was added to the 96-well plate, and the cell numbers were measured using a spectrophotometer at 450 nm absorbance at 2, 12, 24, 36, 48, 60, and 72 h of culture. Wells without cells were served as negative controls.

To examine the migratory activity of MDPSCs (G-CSF gradient 0, 10, 100, and 500 ng/ml), colony-derived DPSCs, pulp CD105<sup>+</sup> cells, and iPS cells, a horizontal chemotaxis assay was performed as described previously<sup>[5]</sup>. The TAXIScan-FL (ECI, Inc., Kanagawa, Japan) was used to detect the real-time horizontal chemotaxis of cells.

2.6. Detection of telomerase activity

Telomerase activity was determined with a Quantitative Telomerase detection kit (Allied Biotech, Inc., Vallejo, CA) according to the manufacturer's instructions, which is based on a PCR-designed telomeric repeat amplification protocol (TRAP). Briefly, MDPSCs, pulp CD105<sup>+</sup> cells, colony-derived DPSCs, and iPS cells were lysed in Lysis Buffer. After centrifugation, the protein concentration of supernatant was measured in each extract using the BCA protein assay kit (Pierce, Rockford, IL). Telomerase activity was detected by Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) using whole-cell extract containing 0.5 μg of protein.

2.7. Real-time RT-PCR

Total RNA was extracted with Trizol (Invitrogen) from MDPCs (G-CSF gradient 0, 10, 100, and 500 ng/ml), pulp CD105<sup>+</sup> cells, DPSCs, and iPS cells. First-strand cDNA was synthesized on the total RNA of these cells by reverse transcription using the ReverTra Ace-s (Toyobo, Tokyo, Japan) after DNase I treatment (Roche Diagnostics, Pleasanton, CA) at 37 °C for 20 min. Real-time RT-PCR was performed at 95 °C for 10 s, 65 °C for 15 s, and 72 °C for 8 s using primers for the stem cell markers, Oct3/4, Nanog, Sox2, Reduced expression (1<sup>st</sup>), Growth and differentiation factor 3 (GDF3), LIN28, Signal transducer and activator of transcription 3 (Stat3) and CXCR4 that were labeled with Light Cycler-Fast Start DNA master SYBR Green 1 (Roche Diagnostics) in a Light Cycler (Roche Diagnostics). To examine the mRNA expression of the angiogenic and neurotrophic factors, real-time RT-PCR amplifications of the granulocyte-monocyte colony-stimulating factor (GM-CSF), matrix metalloproteinase (MMP)-3, vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) were also performed (Supplementary Table 1). The mRNA expression was normalized with β-actin. The RT-PCR products were subcloned into the pGEM-T easy vector (Promega, Madison, WI) and confirmed by sequencing.

2.8. Effect of MDPSCs-conditioned medium

At 60% confluence, the culture medium was switched to DMEM without serum, and the conditioned media from MDPCs (G-CSF gradient 100 ng/ml), pulp CD105<sup>+</sup> cells, and DPSCs were collected 24 h later and concentrated approximately 25-fold by an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-3 membrane (Millipore, Billerica, MA). Protein concentration of the conditioned media was determined by a spectrophotometer at 280 nm absorbance. The experiments were repeated three times (3 lots), and one representative experiment is presented.

The relative mRNA expression of stem cell markers, angiogenic, and neurotrophic factors in dental pulp stem cells mobilized by granulocyte-colony stimulating factor (G-CSF) (MDPSCs) (G-CSF gradient 0, 10, 100, and 500 ng/ml) compared with colony-derived DPSCs (DPSCs), pulp CD105<sup>+</sup> cells and induced pluripotent stem (iPS) cells.

### Table 2

<table>
<thead>
<tr>
<th>G-CSF (ng/ml)</th>
<th>MDPCs (n = 3)</th>
<th>Pulp CD105&lt;sup&gt;+&lt;/sup&gt; cells (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>G-CSF 0 ng/ml</td>
<td>G-CSF 10 ng/ml</td>
</tr>
<tr>
<td>CD24</td>
<td>98.5 ± 0.7</td>
<td>96.2 ± 2.0</td>
</tr>
<tr>
<td>CD31</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>CD44</td>
<td>99.2 ± 0.2</td>
<td>98.9 ± 0.9</td>
</tr>
<tr>
<td>CD73</td>
<td>96.2 ± 4.0</td>
<td>99.0 ± 0.4</td>
</tr>
<tr>
<td>CD90</td>
<td>103.8 ± 0.1</td>
<td>99.5 ± 0.8</td>
</tr>
<tr>
<td>CD105</td>
<td>62.8 ± 6.4</td>
<td><strong>94.5 ± 0.7</strong></td>
</tr>
<tr>
<td>CD146</td>
<td>105.2 ± 2.5</td>
<td>121. ± 4.2</td>
</tr>
<tr>
<td>CXCR4</td>
<td>6.5 ± 2.0</td>
<td><strong>120. ± 1.3</strong></td>
</tr>
<tr>
<td>G-CSFR</td>
<td>20.5 ± 4.7</td>
<td><strong>310. ± 2.3</strong></td>
</tr>
<tr>
<td>CD40</td>
<td>0.4 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CD45</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>CD80</td>
<td><strong>28.3 ± 0.9</strong></td>
<td><strong>28.3 ± 0.9</strong></td>
</tr>
<tr>
<td>CD86</td>
<td>0.5 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>MHC class I</td>
<td>94.7 ± 1.4</td>
<td>92.6 ± 2.5</td>
</tr>
<tr>
<td>MHC class II</td>
<td>0.5 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
</tbody>
</table>

CXCR4, chemokine (C-X-C motif) receptor 4; G-CSFR, granulocyte-colony stimulating factor receptor; MHC, major histocompatibility complex.

<sup>*p < 0.01, **p < 0.05 versus DPSCs.</sup>
(Nacalai tesque, Kyoto, Japan) (final concentration; 100 ng/ml), incubated for 3 h in a humidified 37 °C, 5% CO2 incubator and used as the stimulator in the MLR assay. Autologous PBMCs and allogenic stimulator PBMCs were co-cultured at 10^5 cells per well with a 96-well plate RPMI-1640 without arginine, leucine, lysine, and phenol red (Sigma–Aldrich) supplemented with 5 µg/ml of conditioned medium. The cell numbers were measured using Tetra-color one at 2, 12, 24, and 36 h as described above.

To assess the anti-apoptotic effect of the conditioned medium, NIH3T3 cells were incubated with 500 ng staurosporine (Sigma–Aldrich) in DMEM supplemented with 5 µg/ml of conditioned medium from MDSCPs, pulp CD105+ cells, and DPSCs. As a positive control, G-CSF (100 ng/ml) was added. After 3 h, the NIH3T3 cells were harvested, and the cell suspensions were treated with Annexin V-FITC and propidium iodide (Roche Diagnostics) for 15 min and then analyzed by flow cytometry.

2.9. Maintenance in characteristics of MDSCPs

To evaluate and characterize the stability of MDSCPs, senescence associated (SA)-β-gal staining assay, telomeric repeat amplification protocol (TRAP), real-time RT-PCR, and western blot analysis were performed. SA-β-gal staining assay was performed using Senescence Cells Histochemical Staining Kit (Sigma–Aldrich) according to the manufacturer's instructions. TRAP was described performed as described above. Real-time RT-PCR were performed using primers for senescence related genes (Interleukin-1β (IL-1β), p16, Interleukin-6 (IL-6), Interleukin-8 (IL-8), p21, Growth related oncogene-a (GRoA)) (Supplemental Table 1). Western blot analysis were performed using the anti-p16 antibody (C-20) (1:250) (sc-468, Santa Cruz Biotechnology Inc, CA), anti-p21 antibody (F-5) (1:500) (Santa Cruz), and anti-J-β-actin antibody (RB-9421, Neomarkers, Fremont, CA). The protein bands were detected by immunoblotting (Zeta (Wako)). The 6th, 12th, 25th, and 30th passages of culture of MDSCPs and colony-derived DPSCs were compared (n = 3, each).

2.10. Telomere length analysis

Human MDSCPs (4 × 10^5 cells, 20 µl/site at the 20th passage) were injected into testis or subcutaneous in 6-week-old NOD/SCID (NOB,CD17-Prkdcscid) mice (Faximice Mouse stock #: 001303, The Jackson Laboratory, Bar Harbor, Maine) (n = 8 mice) and in 8-week-old KSN nude (KSN/SLC) mice (Japan SLC, Inc., Hamamatsu, Japan) (n = 15 mice). The mice were sacrificed and all organs were dissected under anesthesia after collecting blood from the post-vena cava for hematological examination. Chromosomal karyotypes were analyzed. The karyotypes were examined in a murine model of hindlimb ischemia[25] in 5-week-old CB17 SCID mice (CleA). The four roots in which only collagen TE was injected were also transplanted as a control. The roots were harvested for histology after 21 days, fixed in 4% paraformaldehyde (PFA) (Sigma–Aldrich) at 4 °C overnight, dehydrated with paraffin wax (Sigma–Aldrich) after demineralization with Kalkiton® (Wako, Osaka, Japan). The paraffin sections (5 μm in thickness) were stained with hematoxylin and eosin (HE). Four sections at 150 µm intervals for 4 roots, each transplanted with four cell populations, were examined for relative amounts of regenerative tissue, as described previously[26]. The ratios of the regenerated areas to the root canal areas were calculated (n = 4 roots). For analysis of matrix formation, each 3 paraffin sections from 4 roots, each transplanted with four cell populations, were also stained with Masson trichrome (Muto pure chemicals Co., Ltd, Tokyo, Japan). For neovascularization and innervation analyses, 5-μm-thick paraffin sections were deparaffinized and stained with Fluorescein Griffonia (Bandeiraea) Simplicifolia Lectin 1/fluorescein-galanthus nivalis (snowdrop) (BS-1 lectin) (20 µg/ml) and PCP9.5 (Ulra Clone Ltd) (1:10,000) respectively[26]. The ratio of the newly formed BS-1 lectin-positive capillaries to the root canal area was calculated using confocal laser microscopy (TCS SP5 conventional inverted microscope, Leica Microsystems, Wetzlar, Germany).

To confirm that the regenerated tissue is functional pulp tissue, western blot analysis of the expression of thyrotropin-releasing hormone degrading enzyme (TRH-DE), a pulp tissue biomarker[27] in the regenerated tissue, was performed 21 days after transplantation of MDSCPs (G-CSF gradient 100 ng/ml) pulp CD105+ cells, and DPSCs. For confirmation, 3 roots, each harvested previously, were embedded in paraffin and the peritoneal cavity tissue of the SCID mice were used as positive and negative controls, respectively. The anti-TRH-DE antibody (N-18) (1:1000) (sc-83177, Santa Cruz Biotechnology Inc, CA) and anti-J-β-actin antibody (RB-9421, Neomarkers, Fremont, CA) were used. The protein bands were detected by Immunostar® (Wako).

For molecular biological analysis, regenerative tissues were further isolated from each of the 3 roots 21 days after transplantation of MDSCPs and DPSCs. The normal pulp tissue from the SCID mice was used as a positive control. Total RNA was isolated, and real-time RT-PCR amplifications were performed using markers for pulp tissue, syntenin-3 and TRH-DE[27], a marker for periodontal ligament and predontoblasts, periostin[28].

2.13. An experimental model of subcutaneous transplantation of the human tooth root

A subcutaneous ectopic transplantation model of the human tooth root was used for evaluation of ectopic pulp regeneration. The human tooth root was cut out, with dimensions of 6 mm in length and 1 mm in width, followed by enblishing and sealing of one end with MTA cement. At the 6-8th cell culture passage, 1 × 10^5 cells from MDSCPs (G-CSF gradient 100 ng/ml), pulp CD105+ cells, and DPSCs were injected into the tooth root with collagen TE (Nitta Gelatin, Osaka, Japan), and each of 4 roots was transplanted subcutaneously into the 5-week-old SCID mice (CB17, CLEA). The four roots in which only collagen TE was injected were also transplanted as a control. The roots were harvested for histology after 21 days, fixed in 4% paraformaldehyde (PFA) (Sigma–Aldrich) at 4 °C overnight, dehydrated, and embedded in paraffin wax (Sigma–Aldrich) after demineralization with Kalkiton® (Wako, Osaka, Japan). The paraffin sections (5 μm in thickness) were stained with hematoxylin and eosin (HE). Four sections at 150 μm intervals for 4 roots, each transplanted with four cell populations, were also stained with Masson trichrome (Muto pure chemicals Co., Ltd, Tokyo, Japan). For neovascularization and innervation analyses, 5-μm-thick paraffin sections were deparaffinized and stained with Fluorescein Griffonia (Bandeiraea) Simplicifolia Lectin 1/fluorescein-galanthus nivalis (snowdrop) (BS-1 lectin) (20 µg/ml) and PCP9.5 (Ulra Clone Ltd) (1:10,000) respectively[26].
2.14. Statistical analyses

Data are reported as means ± SD. *P* values were calculated using Student’s *t*-test and Tukey’s multiple comparison test method in SPSS 21.0 (IBM, Armonk, NY).

3. Results

3.1. The isolation of dental pulp stem cells by the G-CSF mobilization

To optimize the isolation methods for DPSCs subsets by the method of G-CSF-induced stem cell mobilization, the cell numbers seeded in the upper chamber, the incubation time of the G-CSF-containing medium and the final concentration of G-CSF in the lower chamber were examined. At first, we determined that the optimal cell number was 2 × 10^5 cells/100 μl and that the optimal incubation time was 48 h because the number of transmigrated and attached cells in the lower chamber under these conditions was the highest (data not shown). When MDPSCs were isolated with these optimal conditions, the isolation efficiency was 5.0%. The MDPSCs using various concentrations of G-CSF were morphologically similar, containing stellate cells with long processes and spindle-shaped cells almost identical to the pulp CD105+ cells isolated by flow cytometry (Supplementary Fig. 1). The limiting dilution analysis of the third cell culture passage showed that the frequency of colony forming units (CFUs) in MDPSCs was significantly higher compared to colony-derived DPSCs (DPSCs) (88.4 ± 7.9%; 80.4 ± 4.1%, respectively, *p* < 0.05, *n* = 3).

Next, the optimal concentration of G-CSF was determined based on an evaluation of “stemness” of MDPSCs using flow cytometric analysis and real-time RT-PCR analysis. The MDPSCs using various concentration of G-CSF (0, 10, 100, and 500 ng/ml), pulp CD105+ cells and DPSCs were positive for CD29, CD44, CD73 and CD90 and negative for CD31, which are the minimal criteria for MSCs. It is noteworthy, however, that the percentages of CD105, CXCR4 and G-CSFR positive cells were much higher in MDPSCs using 100 ng/ml of G-CSF compared with those in other MDPSCs and DPSCs. The percentages of CXCR4 and G-CSFR positive cells were also higher in the MDPSCs using 100 ng/ml of G-CSF than those in pulp CD105+ cells. These results suggested that the MDPSCs using 100 ng/ml of G-CSF contained the largest number of pulp stem/progenitor cell populations (Table 1). The G-CSF-induced mobilization method for the isolation of DPSCs subsets was repeated over twenty times, resulting in highly reproducible technique. Furthermore, the MDPSCs, pulp CD105+ cells and DPSCs were positive for MHC class I (HLA-A, B, and C), low positive for CD80, CD86, and MHC class II (HLA-Dp, DQ, and DR), and negative for CD40 and CD45. It was suggested that little hematopoietic and immunogenic cells were contained in MDPSCs, pulp CD105+ cells, and DPSCs.

The mRNA expression of the stem cell markers, Oct3/4, Nanog, Sox2, Rex1, GDF3, LIN28, Stat3 and CXCR4 was higher in the MDPSCs using G-CSF (10, 100, 500 ng/ml) compared to the DPSCs and the MDPSCs using serum only (without G-CSF) and was similar to that in pulp CD105+ cells (Table 2). The mRNA expression of the stem cell markers was the highest in the MDPSCs using 100 ng/ml of G-CSF compared to the MDPSCs using other concentrations of G-CSF (Table 2), suggesting that the optimal concentration of G-CSF to isolate MDPSCs may be 100 ng/ml.

The expression of angiogenic and/or neurotrophic factors, GM-CSF, MMP3, VEGF, BDNF, GDNF, NGF and NT-3 was also the highest in the MDPSCs using 100 ng/ml of G-CSF compared to the MDPSCs using other concentrations. The MDPSCs using 100 ng/ml of G-CSF exhibited much higher expression of these factors compared with DPSCs (Table 2), implying that MDPSCs have higher angiogenic/vasculogenic and neurogenic potential. To further characterize the phenotype of MDPSCs, the mRNA expression in MDPSCs (G-CSF gradient 100 ng/ml) was compared with human iPSCs. The expression of Sox2 and CXCR4 mRNA was 182 and 12 times higher, respectively, in iPSCs than in MDPSCs. The MDPSCs, however, expressed other characteristic stem cell markers, including Oct3/4, Nanog, Rex1, GDF3, LIN28 and Stat3, almost the same as iPSCs. On the other hand, the mRNA expression of the angiogenic and/or neurotrophic factors, GM-CSF, MMP3, VEGF, BDNF, GDNF, NGF and NT-3 was much higher in MDPSCs compared to iPSCs (Table 2).

3.2. The multi-lineage differential potential of MDPSCs

The multi-lineage differential potential of MDPSCs (G-CSF gradient 100 ng/ml) was compared with that of DPSCs and pulp CD105+ cells. The MDPSCs (G-CSF gradient 100 ng/ml) formed extensive networks of cords and tube-like structures after only 6 h on the matrigel, as did pulp CD105+ cells. However, no such formation was detected in DPSCs (Fig. 1A–C). Fourteen days after neurogenic induction, clusters of proliferating neurospheres were more prevalent in MDPSCs (G-CSF gradient 100 ng/ml) and pulp CD105+ cells compared with DPSCs (Fig. 1D–F). Following further neuronal induction, neurite outgrowth and the expression of neurofilament found in MDPSCs were similar to those in pulp CD105+ cells (Fig. 1G–I). Neuronal markers, such as neurofilament, neuregulin, and sodium channel, voltage-gated type 1a (Scn1A), showed higher mRNA expression in MDPSCs using 100 ng/ml of G-CSF when compared with the other MDPSCs and DPSCs (Fig. 1J). As for adipogenic induction, the MDPSCs (G-CSF gradient 100 ng/ml) and pulp CD105+ cells but also DPSCs were positively stained by oil red O (Fig. 1K–M), showing expression of the adipogenic markers, aP2 and PPARY mRNA. The MDPSCs using 100 ng/ml of G-CSF, however, had the highest adipogenic potential (Fig. 1N). Finally, 28 days after osteogenic induction, the mineralized matrix was stained by alizarin red (Fig. 1O–Q), and the osteoblastic marker, osteocalcin was similarly expressed in all cell populations (Fig. 1R).

3.3. The biological characteristics of MDPSCs

Telomerase activity was 3.7 times and 4.1 times higher in MDPSCs at the 7th cell culture passage compared with DPSCs and pulp CD105+ cells, respectively, and 0.6 times higher compared with iPSCs (Fig. 1S). The cumulative cell number of MDPSCs was much higher, and the proliferative life span of MDPSCs was longer than that of DPSCs (Fig. 1T). Proliferation with human serum was higher in MDPSCs than in DPSCs, but there were no significant differences among the MDPSCs using 10, 100, 500 ng/ml G-CSF concentrations (Fig. 1U). The G-CSF-induced migratory activity was much higher in MDPSCs than that in the DPSCs, and the MDPSCs using 100 ng/ml of the G-CSF exhibited the highest activity (Fig. 1V).

The conditioned medium (CM) of the MDPSCs (G-CSF gradient 100 ng/ml) was significantly more effective on the proliferation (Fig. 1W) and migratory activity in NIH3T3 cells (Fig. 1X) compared with the CM of pulp CD105+ cells and DPSCs. The MLR assay showed significantly enhanced immunosuppression by the CM of MDPSCs (G-CSF gradient 100 ng/ml) compared with the CM of DPSCs (Fig. 1Y). The survival rate of NIH3T3 cells was also significantly more enhanced by the CM of the MDPSCs (G-CSF gradient 100 ng/ml) compared with the CM of DPSCs (Fig. 1Z). These results suggested that the MDPSCs using 100 ng/ml of G-CSF exhibited the biological properties with the most powerful regenerative potential among the MDPSCs using various concentrations. We therefore determined the optimal concentration of G-CSF to be 100 ng/ml.
3.4. Maintenance of the stem properties and safety evaluation of MDPSCs

To examine the stability of MDPSCs after prolonged ex vivo culture, expression of cellular senescence markers in the MDPSCs was compared to that in the DPSCs at the 6th, the 12th, and the 30th passages. Both MDPSCs and DPSCs at the 6th passage and MDPSCs at the 12th passage were not stained for SA-β-gal, but 3.1% of DPSCs at the 12th passage was positively stained. At the 30th passage, the SA-β-gal positive cells were detected in 60% of DPSCs, but only in 13% of MDPSCs (Fig. 2A–C). The telomerase activity was decreased in DPSCs at the 25th passage, but showed almost the same level in MDPSCs at the 25th passage as that in MDPSCs at the 6th passage (Fig. 2D). The mRNA expression of IL-1β, p16, IL-6, and IL-8 was highly up-regulated in the DPSCs at the 30th passage, but not in the MDPSCs (Fig. 2E). Western blot analysis revealed that expression of p16, and p21 was significantly increased in DPSCs at the 25th passage compared to those at the 6th passage, but almost the same level in MDPSCs between the 6th and the 25th passages (Fig. 2F). These results indicated the stability of MDPSCs in long-term in vitro expansion.

Furthermore, intratesticular or subcutaneous injection of MDPSCs in immunodeficient SCID mice resulted in no teratoma formation (Fig. 2G) and hematological abnormality. There were also no chromosomal abnormalities/aberrations in the karyotype of MDPSCs at the 20th passage (Fig. 2H).

Fig. 2. Maintenance of the stem cell properties, tumorigenicity assay, and Q-band karyotype analysis of DPSCs mobilized by G-CSF (MDPSCs) (G-CSF gradient 100 ng/ml). (A, B) Senescent associated (SA)-β-gal staining. (C) Percentage of SA-β-gal-positive cells at the 6th, the 12th, and the 30th passages. **p < 0.01. (D) Relative telomerase activity in MDPSCs and DPSCs at the 6th, the 25th and the 30th passages. **p < 0.01. (E) Relative mRNA expression of senescence related genes in MDPSCs and DPSCs at the 6th and the 30th passages. *p < 0.05. (F) Protein level of p16 and p21 in MDPSCs and DPSCs at the 6th and the 25th passages. (G) Tumorigenicity assay. No tumorigenicity in the testis 16 weeks after transplantation of MDPSCs was observed. (H) Q-banding analysis for MDPSCs at the 20th passage showing normal karyotype. The experiment was repeated three times (3 lots) and one representative experiment was presented.
3.5. Neovascularization in the ischemic hindlimb after MDPSCs transplantation

Fourteen days after transplantation, quantitative analyses of blood flow and capillary density were performed. Laser Doppler imaging revealed that blood flow was significantly increased approximately 1.8, 2.0 and 2.2 times more in the MDPSCs transplantation compared with DPSCs, iPS cells, PBS control without cells, respectively (Fig. 3A–F). The capillary density in the ischemic region transplanted with MDPSCs increased 1.7, 4.3 and 7.4 times.

Fig. 3. Neovascularization in the ischemic hindlimb 14 days after transplantation of dental pulp stem cells (DPSCs) mobilized by granulocyte-colony stimulating factor (G-CSF) (MDPSCs), pulp CD105+ cells, colony-derived DPSCs (DPSCs), and induced pluripotent stem cells (iPS cells). (A–E) Laser Doppler imaging. Accelerated blood flow (arrows). (F) Quantification of blood flow in the ischemic versus normal limbs obtained from four mice in each group. *p < 0.05. (G–K) Immunostaining of Fluorescein Griffonia (Bandeiraea) Simplicifolia Lectin 1/flourescin-galanthus nivalis (snowdrop) lectin (BS-1 lectin) in the ischemic hindlimb. (L) Quantification and statistical analysis of the capillary density in the ischemic region using serial sections. Data are expressed as means ± SD of 4 determinations. *p < 0.05, **p < 0.01. The experiments were repeated three times (3 lots), and one representative experiment is presented. (M–P) Localization of DiI-labeled transplanted cells and newly formed capillaries stained by BS-1 lectin.
Fig. 4. Regeneration of pulp tissue after ectopic tooth transplantation in severe combined immunodeficiency (SCID) mice. Dental pulp stem cells (DPSCs) mobilized by granulocyte-colony stimulating factor (G-CSF) (MDPSCs), pulp CD105+ cells, colony-derived DPSCs (DPSCs), and induced pluripotent stem cells (iPS cells) were injected into the emptied root canals. (A–D) Hematoxylin and Eosin (HE) staining. (E–H) Masson trichrome staining. (I–L) Immunostaining with BS-1 lectin. (M) Ratio of the regenerated area to the root canal.
higher than that with DPSCs, iPSCs cells and PBS control, respectively (Fig. 3G–L). There was no significant difference in blood flow or capillary density between MDPSCs and pulp CD105+ cells. Dilabeled MDPSCs, pulp CD105+ cells, and DPSCs were not co-localized with BS-1 lectin stained blood vessels (Fig. 3M–O), implying their trophic effect on neovascularization. On the other hand, iPSCs cells were co-localized (Fig. 3P), indicating their differentiation potential into endothelial cells.

3.6. Ectopic pulp regeneration in the tooth root after MDPSCs transplantation

We next evaluated the pulp regeneration potential of human MDPSCs in an experimental model of ectopic tooth root transplantation in SCID mice. Pulp-like tissue with a well-organized vasculature was regenerated in the tooth root 21 days after transplantation of MDPSCs, pulp CD105+ cells, DPSCs and iPSCs cells (Fig. 4A–D). Masson trichrome staining, however, revealed that fibrous matrix formation was observed in part of the DPSCs and iPSC cells transplantation (Fig. 4G, H). Statistical analysis showed that the regenerated area was significantly larger (1.2-fold and 1.5-fold, respectively) in the MDPSCs transplantation compared to the DPSCs and the iPSC cells transplantations, and was similar to the CD105+ cells transplantation (Fig. 4M). Immunofluorescence staining analysis with BS-1 lectin demonstrated neovascularization in the regenerated pulp tissue after all the cell transplantations (Fig. 4I–L) but little neuronal process stained with PGP9.5 antibody (data not shown). Statistical analysis showed that the vasculization areas were significantly larger (1.3-fold, 2.3-fold, respectively) in the transplantation of MDPSCs compared to DPSCs and iPSCs cells, and was similar to that of pulp CD105+ cells (Fig. 4N).

The regenerated tissue was further analyzed by western blot analysis and real-time RT-PCR analysis. The protein expression of TRH-DE, a biomarker of pulp tissue, was significantly higher in the regenerated tissue of the MDPSCs transplantation than in those of the DPSCs transplantation and the pulp CD105+ cells transplantation. There was no signal in the peritoneal cavity tissue (Fig. 4O). The expression of Syndecan 3 and TRH-DE was similar between the regenerated tissue of the MDPSCs transplantation and normal mouse pulp tissue (Fig. 4P). The expression of Periostin was higher in the regenerated pulp tissue of the DPSCs transplantation compared to that of the MDPSCs transplantation (Fig. 4P).

4. Discussion

DPSCs subsets have a higher migration activity compared with unfractionated pulp cells [5,23]. The mobilization of bone marrow hematopoietic stem cells in vitro by G-CSF has been previously reported [29,30]. Thus, in this study, for the first time we have developed a safe and efficacious method to isolate DPSCs subsets based on G-CSF-induced stem cell mobilization. A special device was developed in which the transmembrane of the upper chamber was chemically treated to prevent cell attachment and increase the number of transmigrating cells. The optimal isolation conditions for MDPSCs were determined as follows: the plating cell number in the upper chamber, 2 × 10^6 cells/100 μl, the final concentration of G-CSF in the lower chamber, 100 ng/ml, and the incubation time with G-CSF, 48 h. For the migration of bone marrow hematopoietic stem cells, 100 ng/ml of G-CSF was added for only 80 min [29]. In our system, however, the migrating cell number was very low at 12 h and gradually increased to a maximum at 48 h. The MDPSCs increased proliferation but did not differentiate into odontoblasts by exposure to 100 ng/ml of G-CSF (data not shown), suggesting optimal conditions. The material used for chemically modification of the transmembrane has been already approved for clinical use in the Japanese Pharmaceutical affairs law with safety confirmation. Thus, these findings indicated that MDPSCs can be isolated efficiently and safely from a small number of cells using this device under the optimal conditions.

Pulp CD105+ cells isolated by flow cytometry are more enriched for stem/progenitor cells, having a higher angiogenic and neurogenic potential, compared with DPSCs [3,5]. Thus, MDPSCs were compared with DPSCs, pulp CD105+ cells and iPSCs cells for cell phenotype, biological characteristics, and regenerative potential in an ischemic hindlimb model and an ectopic tooth root model. The cell surface marker expression of CD105, and the mRNA expression of stem cell markers such as Oct3/4 and Nanog in MDPSCs were similar to those in pulp CD105+ cells, suggesting a stemness phenotype of MDPSCs similar to that of pulp CD105+ cells. MDPSCs exhibited a higher migratory activity and higher expression of angiogenic/neurotrophic factors in vitro and greater angiogenic/vasculogenic and regenerative potential in the two in vivo models compared to DPSCs and iPSCs cells. The transplanted MDPSCs were localized in the vicinity of newly formed vessels and not differentiated directly into endothelial cells as the transplanted pulp CD105+ cells and DPSCs. On the other hand, the transplanted iPSC cells differentiated into endothelial cells in the ischemic hindlimb model. A similar report has been reported in mouse ischemic brain model that transplantation of iPSCs can provide neural cells and vascular endothelial cells and is associated with teratoma formation [31]. Vascular progenitor cells derived from iPSCs cells also successfully recover blood flow after transplantation in the same model. The possibility of the contamination of undifferentiated iPSCs cells and safety concern for isolation of the differentiated vascular progenitor cells from undifferentiated iPSCs cells by flow cytometry, however, need to be overcome [32]. On the other hand, the absence of abnormalities/aberrations in karyotype and no tumorigenicity of MDPSCs in an immunodeficient mouse at the 20th passage of culture were also demonstrated. Furthermore, G-CSF and CXCR4 are more highly expressed in MDPSCs compared to DPSCs, which have the advantage of a synergistic/additive effect with G-CSF [33] and SDF-1 [5]. After injection, MDPSCs may be mobilized and home to the injured sites of the body releasing G-CSF [20,21,34] and/or SDF-1 [35,36]. Thus, these findings on safety and efficacy suggest that MDPSCs are a superior cell source compared to DPSCs and iPSCs cells for stem cell therapy. In addition, the MDPSCs can undergo 30 to 40 passages, whereas the adult bone marrow MSCs can grow identically for 6–10 passages [37]. MDPSCs have demonstrated the stability of the cell phenotype after long-term expansion including fewer staining with SA-β-gal, lower expression of mRNA and proteins of senescence markers, p16 and p21 etc. and higher telomerase activity compared with DPSCs. These results assume large-scale produced MDPSCs as “cell medicine” for safety evaluation and clinical application. Therefore, G-CSF-induced mobilization might be an efficient and safety method to establish standardized manufacture guideline for the isolation of MSCs to enrich a subset of
of DPSCs secreting high levels of trophic factors and endowed with high regenerative potential. The cell banking of MDPSCs for autologous transplantation will be served with a guarantee of high quality control.

Expression of the characteristic stem cell markers Sox2, Oct3/4, Nanog, Rex1, GDF3 and CXCRI4 was higher in MDPSCs compared to DPSCs. Interestingly, Oct3/4, Nanog, Rex1, and CDF3 were similarly expressed in MDPSCs and iPSCs. iPSCs are generated from pulp stromal cells [38,39], and the efficiency of iPSC generation is higher than that from dermal fibroblasts [39]. Thus, MDPSCs might be a preferable alternative cell source for the generation of iPSCs with the potential for higher efficiency due to the higher expression of Sox2 and Oct3/4 compared with DPSCs.

5. Conclusions

An isolation of DPSCs subsets based on their migratory response to 100 ng/ml of G-CSF is safe and efficacious method. CD105, CXCRI4 and G-CSFR positive stem cells are enriched in MDPSCs having high proliferative and migration activities, and trophic effect including anti-apoptosis, accelerating migration and immunomodulation. MDPSCs are useful cell source for cell therapy not only in regenerative endodontics but also in other regenerative medicine applications for ischemic diseases and neuronal injury with higher regenerative potential compared with DPSCs. MDPSCs isolated using reagents compatible with good manufacturing practice (GMP) may be of utility for potential clinical applications in the near future.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

We thank Mr. Masaaki Shimagaki from Toray Industry Inc. for supplying the chemically treated transmembrane. This work was supported by the Budget for promoting science and technology in Japan, which directly follows the policy of the Council for Science and Technology Policy (CSTP), chaired by the Prime Minister (M.N.), and the Research Grant for Longevity Sciences (23–10) from the Ministry of Health, Labour and Welfare (M.N.). The authors have no potential conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.08.011.

References


