Mobilized Dental Pulp Stem Cells for Pulp Regeneration: Initiation of Clinical Trial

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Abstract

Stem cell therapy is a potential strategy to regenerate the dentin-pulp complex, enabling the conservation and restoration of functional teeth. We assessed the efficacy and safety of pulp stem cell transplantation as a prelude before the initiation of clinical trials. Granulocyte-colony stimulating factor (G-CSF) induces subsets of dental pulp stem cells to form mobilized dental pulp stem cells (MDPSCs). Good manufacturing practice is a prerequisite for the isolation and expansion of MDPSCs that are enriched in stem cells, expressing a high level of trophic factors with properties of high proliferation, migration, and antiapoptotic effects and endowed with regenerative potential. The quality of clinical-grade MDPSCs was assured by the absence of abnormalities/aberrations in karyotype and the lack of tumor formation after transplantation in immunodeficient mice. Autologous transplantation of MDPSCs with G-CSF in pulpectomized teeth in dogs augmented the regeneration of pulp tissue. The combinatorial trophic effects of MDPSCs and G-CSF on cell migration, antiapoptosis, immunosuppression, and neurite outgrowth were also confirmed *in vitro*. Furthermore, MDPSCs from the aged donors were as potent as the young donors. It is noteworthy that there were no significant age-related changes in biological properties such as stability, regenerative potential, and expression of the senescence markers in MDPSCs. On the other hand, autologous transplantation of MDPSCs with G-CSF induced less regenerated pulp tissue in the aged dogs compared with the young dogs. In conclusion, the preclinical safety, feasibility, and efficacy of pulp regeneration by MDPSCs and G-CSF were established. Therefore, the standardization and establishment of regulatory guidelines for stem cell therapy in clinical endodontics is now a reality. *(J Endod 2014;40:S26–S32)*

Key Words

Angiogenesis, CD105, dentinogenesis, mobilized dental pulp stem cells, neurogenesis, preclinical trial, pulpectomy, pulp regeneration, preclinical trial, stem cell isolation method

In an aging society, the elderly face challenges in the maintenance of health including dental health, and the dental profession has emphasized the importance of the preservation of teeth for optimal quality of life. Dental pulp has multiple functions in the homeostasis of teeth, and maintenance of the function of pulp tissue is critical for the longevity of teeth. The ideal approaches for endodontic treatment are conservation of healthy tooth structure, prevention of microleakage from pulp cavity, and maintenance of the properties and mechanical strength of the tooth structure. Stem cell therapy with pulp stem/progenitor cells is a useful strategy to regenerate the dentin-pulp complex (1). Innervation and vasculature of dental pulp are intimately associated in pulp homeostasis, and both angiogenesis/vasculogenesis and neurogenesis are essential for pulp regeneration. We have previously shown complete pulp regeneration harnessing $CD105^+$ dental pulp stem cells (DPSCs) and pulp $CD31^-$ side population (SP) cells with stromal cell–derived factor 1 (SDF-1) in a canine pulpectomy model (2–4). The high regenerative potential of SP cells including angiogenesis/vasculogenesis and neurogenesis/reinnervation after transplantation in models of hind limb (2, 5, 6) and brain ischemia (6–8) and ectopic tooth root transplantation was shown (6). Thus, certain DPSC subsets might be useful for cell-based therapy (9–13). For clinical use, good manufacturing practice (GMP) is a prerequisite for the isolation of DPSC subsets. However, the safety of these $CD105^+$ cells and $CD31^-$ SP cells isolated by flow cytometry has not been established because there are still no GMP-grade flow cytometers. Another isolation method using GMP-grade immunomagnetic beads is not suitable for human DPSC subsets because a large number of the primary pulp cells is needed. The costs will also be prohibitive if CD105 magnetic beads are specially made to order for pulp stem cell isolation. Thus, there are no methods in place for flow cytometer or immunomagnetic beads to isolate GMP-grade DPSC subsets for clinical use.

In this investigation, we devised a method to isolate GMP-grade DPSC subsets using optimized G-CSF–induced mobilization (14) that is cost-effective and leading to safe and efficacious isolation from a small number of pulp cells. Here we present the regenerative potential of mobilized dental pulp stem cells (MDPSCs) in comparison with DPSCs in a hind limb ischemic model and an ectopic tooth root transplantation model. Potential clinical applications of MDPSCs were assessed by the safety and efficacy of pulp stem cell transplantation as a prelude for the

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initiation of clinical trials. Furthermore, MDPSCs from aged donors were compared with MDPSCs from young donors with special reference to the stability and expression of senescence markers. The regenerative potential of aged MDPSCs was also examined in models of ischemic hind limb and ectopic tooth root, showing that it is independent of aging.

Isolation and Characterization of Human DPSC Subsets by the G-CSF–induced Mobilization

We optimized a method for isolating human DPSC subsets using G-CSF–induced stem cell mobilization, yielding MDPSCs (14). Transwell (Corning, Tewksbury, MA), with a chemically treated membrane, was used as the upper chamber to prevent cell attachment and was inserted into 24-well tissue culture plates as the lower chamber to which transmigrated cells from the upper chamber by G-CSF gradient attached to grow (Fig. 1). The optimized number of cells seeded in the upper chamber was 2×10^4 cells/100 μL, and the optimized incubation time of the G-CSF–containing medium was 48 hours. The concentration of G-CSF (100 ng/mL) resulted in an optimum based on an evaluation of "stemness" of MDPSCs using flow cytometric analysis of CD105, CXCR4 (chemokine [C-X-C motif] receptor 4), and granulocyte-colony stimulating factor receptor (G-CSFR) and real-time reverse-transcription polymerase chain reaction analysis of the stem cell markers *Oct4A*, *Nanog*, *Sox2*, *Rex1*, *GDF3*, *LIN28*, *Stat3*, and *CXCR4*. MDPSCs using 100 ng/mL G-CSF exhibited a much higher expression of angiogenic and/or neurotrophic factors (ie, granulocyte-macrophage colony-stimulating factor [GM-CSF], matrix metalloproteinase 3 [MMP3], vascular endothelial growth factor [VEGF], brain-derived neurotrophic factor [BDNF], glial cell derived neurotrophic factor [GDNF], nerve growth factor [NGF], and neurotrophin 3 [NT-3]) compared with colony-derived DPSCs without isolation, implying that MDPSCs may have higher angiogenic/vasculogenic and neurogenic potential. Messenger RNA (mRNA) expression in MDPSCs was further compared with human induced pluripotent stem cell (iPS) cells. *Sox2* and *CXCR4* mRNA was expressed 182 and 12 times higher, respectively, in iPS cells compared with MDPSCs. Other stem cell markers (ie, *Oct4A*, *Nanog*, *Rex1*, *GDF3*, *LIN28*, and *Stat3*) were similarly expressed in MDPSCs as those in iPS cells. The expression of the following angiogenic and/or neurotrophic factors was much higher in MDPSCs compared with iPS cells: GM-CSF, MMP3, VEGF, BDNF, GDNF, NGF, and NT-3.

The multilineage differential potential of MDPSCs was compared with that of DPSCs (14). The MDPSCs formed extensive networks of cords and tubelike structures as early as 6 hours on Matrigel (BD Biosciences, San Jose, CA) although no such formation was detected in DPSCs. The formation of neurospheres, detached proliferating clusters of cells, was more prevalent in MDPSCs compared with DPSCs 14 days after neuronal induction. The neurosphere cells from both cells were dissociated and plated to permit cell adhesion. Most of the cells induced from MDPSCs were immunoreactive for neurofilament, having a few long, thin processes with neuronal morphology 10 days after induction, although some of the cells induced from DPSCs were immunostained with neurofilament. Neuronal markers, such as neurofilament, neuromodulin, and sodium channel, voltage-gated type 1α (Scn1A), showed higher mRNA expression in MDPSCs compared with DPSCs. Both MDPSCs and DPSCs were positively stained with oil red O, showing mRNA expression of the adipogenic markers adipocyte Protein 2 (aP2) and peroxisome proliferator-activated receptor 4 (PPARγ) in both cell types, indicating adipogenic induction. Finally, 28 days after osteogenic induction, the mineralized matrix was similarly stained by alizarin red in MDPSCs and DPSCs, expressing the osteoblastic marker osteocalcin.

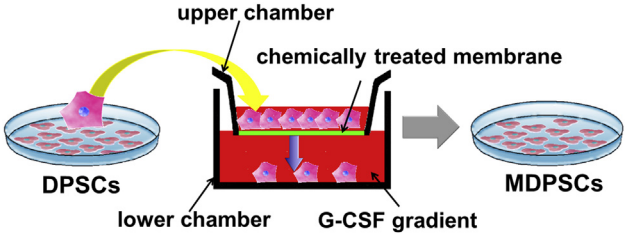

Figure 1. Schematic diagrams demonstrating the method of isolation of mobilized dental pulp stem cells (MDPSCs) from colony derived dental pulp stem cells (DPSCs).

Next, the biological characteristics of MDPSCs were examined (14). The proliferation and migration potential was higher in MDPSCs compared with DPSCs. The cumulative cell number of MDPSCs was much higher, and the proliferative life span of MDPSCs was longer than that of DPSCs. The telomerase activity was 3.7 times and 0.6 times higher in MDPSCs at the 7th passage of culture compared with DPSCs and iPS cells, respectively. The conditioned medium (CM) of MDPSCs more significantly stimulated the proliferation and migration activities of NIH3T3 cells compared with the CM of DPSCs. The mixed lymphocyte reactions (MLR) assay showed significantly enhanced immunosuppression by the CM of MDPSCs compared with the CM of DPSCs. The antiapoptotic effect of the CM of MDPSCs was also higher compared with the CM of DPSCs.

Furthermore, the stability of MDPSCs after prolonged culture was shown (14). A cellular senescence marker, senescence-associated beta-galactosidase, was expressed in 13% of MDPSCs even at the 30th passage of culture, in contrast to that in 60% of DPSCs at the same passage. The telomerase activity in MDPSCs was almost the same level at the 25th passage as the 6th passage although it was decreased in DPSCs at the 25th passage compared with the 6th passage. The expression of p16 and p21 was almost the same level in MDPSCs between the 6th and the 25th passages. On the contrary, the increased expression of those senescence markers was shown in DPSCs at the 25th passage.

The safety of MDPSCs was shown by a subcutaneous or intratesticular injection of MDPSCs in immunodeficient severe combined immunodeficiency (SCID) mice, resulting in no teratoma formation (14). No chromosomal abnormalities/aberrations were detected in the karyotype of MDPSCs at the 20th passage by Q-banding karyotype analysis.

To evaluate whether MDPSCs generate new vessels *in vivo*, [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate] (DiI)-labeled MDPSCs were transplanted in an experimental model of ischemia in the hind limb of a mouse (14). Fourteen days after transplantation, laser Doppler imaging revealed an increase in blood flow in transplantation of MDPSCs compared with transplantation of DPSCs and control phosphate buffered saline injection. Capillary density in the muscles of the ischemic hind limb was increased in MDPSC transplantation to a greater extent compared with DPSC transplantation and phosphate buffered saline control. DiI-labeled MDPSCs and DPSCs were not colocalized with BS-1 lectin (Griffonia [Bandeiraea] Simplicifolia lectin)–stained blood vessels, implying their trophic effect on neovascularization.

The pulp regeneration potential of human MDPSCs was shown in an experimental model of ectopic tooth root transplantation in SCID mice (14). Pulplike tissue with a well-organized vasculature system was regenerated in the tooth root 21 days after transplantation of