Selection of optimal passage of bone marrow-derived mesenchymal stem cells for stem cell therapy in patients with amyotrophic lateral sclerosis

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Abstract

Mesenchymal stem cells (MSCs) obtained from bone marrow (BM) are currently used as an alternative therapy in amyotrophic lateral sclerosis (ALS) patients. Selection of optimal passages of autologous BM-derived MSCs during long-term in vitro expansion is important for clinical trials in patients with ALS. We isolated and expanded MSCs from the BM of eight ALS patients to analyze the growth kinetics, differentiation potential, cellular surface antigen expression, karyotype modifications and secretion of various cytokines during long-term culture. The morphology and size of the cells changed from small and spindle-like cells to large and polygonal types in later passages. The growth rate of the MSCs was highest in the third passage, followed by a gradual decrease. There were no special modifications of cell surface antigens or the karyotype of the MSCs from the first to the tenth passage. MSCs in the fourth passage were differentiated into adipocytes, osteocytes and chondrocytes. When we analyzed the cultured media of MSCs at the third, fifth, seventh and ninth passages, IL-6, VEGF and IL-8 showed high expression, with more than 50 pg/10,000 cells at these passages; however, their expression progressively decreased with additional passages. In addition, secretion of IL-15, GM-CSF, IL-10, PDGF-bb, G-CSF, IL-1β, basic FGF and IFN-γ gradually decreased over prolonged culture. We suggest that MSCs at earlier passages are more suitable for stem cell therapy in ALS patients because of their stability and more potent anti-inflammatory and neuroprotective properties.

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MSCs [23]. Considering these observations, it is clear that a number of parameters must be delineated for isolating and expanding MSCs as well as selecting an optimal passage number before the experimental and clinical use of MSCs originating from BM of ALS patients.

In this study, we analyzed the growth kinetics, differentiation potential, cellular surface antigens, karyotype modifications and secretion of various cytokines and growth factors during long-term in vitro expansion of MSCs obtained from the BM of ALS patients. In addition, we attempted to select the optimal passages of expanding MSCs in terms of maximal anti-inflammatory and neuroprotective properties for stem cell therapy in patients with ALS. Eight BM samples were collected from sporadic ALS patients who were undergoing autologous stem cell therapy for ALS. According to the El Escorial revised criteria, clinically probable and definite ALS patients were included. In addition, patients who had a severe clinical form of ALS were included because this clinical trial was performed to assess the safety of the therapy in ALS patients with various conditions (Supplementary Table 1).

After securing sufficient MSCs for therapeutic use, the remaining MSCs were analyzed in this study. All patients provided informed consent. This study was approved by the Institutional Review Board for Human Investigation of the Hanyang University Hospital as part of a clinical trial protocol and by the Korean Food and Drug Association as an emergent clinical trial.

MSCs from the BM of ALS patients were isolated as previously described [13]. To expand the adherent cells, the cells were detached with 0.25% trypsin/ethylenediaminetetraacetic acid for 3 min at 37 °C to completely remove the cells and expand for successive passages until they reached 80–90% confluence.

For adipogenic differentiation, cells at the fourth and ninth passages were plated at 500 cells/cm² and grown for 24 h. They were then incubated in adipogenic medium ( Lonza, Basel, Switzerland). The medium was replaced every 3 days for 16 days. Cultures were washed with PBS, fixed in 10% buffered formalin, and stained with fresh Oil Red O solution (Fisher Scientific, Leicestershire, UK). For osteogenic differentiation, cells at the fourth and ninth passages were plated at 500 cells/cm² and grown for 24 h. They were then incubated in osteogenic medium ( Lonza). The medium was replaced every 3–4 days for 21 days. Cultures were washed with PBS and fixed in 10% buffered formalin. They were incubated with anti-collagen I antibody (Acris Antibodies, Herford, Germany) and stained with avidin-biotin conjugate complex. The areas expressing collagen I appeared brown. For chondrogenic differentiation, 250,000 MSCs at the fourth and ninth passages were transferred into 15-mL polycarbonate tubes and centrifuged at 150 × g for 5 min. The cell pellets were then cultured in chondrogenic medium ( Lonza) and fed every 2–3 days by replacing the medium in each tube. The pellets were harvested after 28 days in culture, washed with PBS, fixed in 10% buffered formalin, and stained with Artisan® Masson’s Trichrome Stain Kit (DakoCytomation, Glostrup, Denmark).

To determine the number of cumulative population doublings (PDs) after primary culture, MSCs were seeded at a density of 4000/cm² from the first passage to the 13th passage. At confluence, the cells were trypsinized and counted. The number of PDs was determined using the formula log N/log 2, where N is the cell number in the confluent monolayer divided by the initial number of cells seeded [23].

At each passage, the cells were trypsinized and washed twice with PBS/2% FBS. The cells were labeled with CD29-FITC (Serotec, Oxford, UK), CD44-FITC (DakoCytomation), CD105-FITC (Serotec), CD34-PE (Serotec), CD45-PE (DakoCytomation), HLA-DR-FITC (Serotec) or CD73-PE (BD Biosciences Pharmingen, CA, USA). Labeled cells were analyzed by FACSCalibur (BD Biosciences Pharmingen). The percent of positive cells was calculated using the cells stained with IgG1 FITC/PE as a negative control.

To observe metaphase, the cells were treated with colcemid (InvitrogenTM, CA, USA) for 2 h and then combined in 1% sodium citrate at 37 °C for 30 min. The cells were fixed in a solution of methanol:acetic acid (3:1) at 4 °C for 30 min. We analyzed 20–30 metaphases after GTG banding using the Applied Imaging CytoVision automated metaphase scanning system (Genetix Ltd., Queensway, UK).

At each passage, MSCs were seeded at a density of 4000/cm² in Mesenchymal Stem Cell Growth Medium (MSCGM; Lonza) and maintained at 37 °C in an atmosphere of 5% CO₂. Supernatants from the MSCs cultured in MSCGM were collected at the third, fifth, seventh and ninth passages and were analyzed by the Bio-Plex Human Cytokine 27-Plex Panel (Bio-Rad, CA, USA) according to the manufacturer’s instructions. A total of 50 μL of the beads were applied to each well of the filter-bottomed microplate, and 50 μL of standards or the cell culture supernatants were then added and incubated for 30 min at room temperature in the dark. The microplate was washed twice with 100 μL of wash buffer. A total of 25 μL of detection antibody was added to each well, and the plate was incubated for 30 min at room temperature in the dark. The wells were then washed twice with 100 μL of wash buffer, and 50 μL of streptavidin-PE in Assay buffer A was added to the wells. The plate was then incubated for 30 min at room temperature in the dark and washed twice with 100 μL of wash buffer. The beads in each well of the plate were resuspended by adding 125 μL of wash buffer and read within 90 min using a Luminex® 200TM (Luminex Corporation, TX, USA).

Growth kinetics were analyzed using OriginPro 7.5 (OriginLab Corporation, MA, USA). All values are expressed as the mean ± standard deviation (S.D.). Statistical analysis was performed using SPSS 13.0 (SPSS Inc., IL, USA). Data were tested using one-way ANOVA followed by the Tukey’s HSD post hoc test. P-values <0.05 were considered significant.

At 3 days after seeding, MSCs, which appeared as adherent fibroblast-like cells, were observed in all samples (Fig. 1A). After 10–12 days, the adherent cells were subcultured, and over 95% of the cells cultured at the first passage possessed spindle-like morphology (Fig. 1A). MSCs isolated from the eight ALS patients did not show morphological differences between patients (data not shown). MSCs from the early passages (first, third and fifth) contained rapidly proliferating spindle-like cells, whereas the cells from the seventh and especially the ninth passages consisted primarily of large, slowly dividing cells (Fig. 1A).

MSCs from the fourth and ninth passages were analyzed for adipogenic, osteogenic and chondrogenic differentiation (Fig. 1B). Adipogenic differentiation of the cells at the fourth passage was determined based on the accumulation of Oil Red O-stained fat droplets compared to control cells. We induced fourth passage MSCs to osteogenic differentiation, and most of the differentiated cells expressed collagen I in contrast to control cells, which rarely expressed collagen I. The fourth passage cells were pelleted into micromasses and then differentiated in chondrogenic medium. Chondrogenic differentiation was demonstrated via lacunae formation and collagen expression. Although ninth passage cells from the same patient were induced under the same conditions to differentiate into adipocytes, osteocytes and chondrocytes, no differentiation was observed (data not shown).

MSCs isolated from eight ALS patients were expanded from passage 1 to passage 13. In all subsequent experiments, the cells were subcultured when they reached 80–90% confluence.

As indicated in Fig. 2A, there was a large variation in PDs among cells obtained from different patients upon subculture when not...
accounting for the age of the patient. Comparison of the growth rates between the middle-aged (patients 1–4) and elderly (patients 5–8) patients showed a significant difference in PDs in the early passages. The middle-aged patients ranged in age from 35 to 45 years, and the elderly patients ranged in age from 60 to 63 years. In the middle-aged patients, the mean number of cumulative PDs was 12.65 ± 1.1 at the fourth passage for 41.75 ± 10.14 days, while the mean number of cumulative PDs was 10.59 ± 1.52 for 41.25 ± 5.56 days in the elderly.

The mean number of PDs in the third passage was 3.15 ± 0.65, which was the highest value observed among the 13 passages (Fig. 2B). MSCs from the first to tenth passages, not including the third passage, showed mean PDs of 2–3, and the mean PD numbers gradually decreased as the passage number increased.

Antigen expression (with ranges) in MSCs isolated from seven patients was analyzed at each passage (Supplementary Table 2). At the first passage, cells were negative for CD34, CD45 and HLA-DR, with antigen expression detected in less than 5% of cells (the mean values were 2.23 ± 2.48%, 4.48 ± 3.04% and 3.32 ± 3.83%, respectively). However, the cells showed high expression of CD29 (99.68 ± 0.17%), CD44 (98.17 ± 2.17%), CD73 (99.55 ± 0.34%) and CD105 (98.86 ± 0.89%). During subculture of the cells from the first to the tenth passage, we found positive antigen expression for CD29, CD44, CD73 and CD105 in an average of 95% of cells. On the contrary, less than 6% of the cells expressed CD34, CD45 and HLA-DR. There were therefore no remarkable changes in antigen expression of MSCs after successive subculture.

Analysis of telomere length in MSCs from patients 3 and 7 revealed that telomere length at the third passage was longer than that at the fifth, seventh and ninth passages (Supplementary Fig. 1).

MSCs isolated from eight ALS patients were analyzed from the second to the tenth passages, and no chromosomal alterations were observed (data not shown).

**Fig. 1.** Change of MSC morphology and differentiation of MSCs. Long-term culture of MSCs affects cell morphology and size. (A) The size and the morphology of the cells transitioned from small and spindle-like to large and polygonal during long-term culture. Yellow arrows indicate a polygonal morphology with evident filaments in the cytoplasm. (B) MSCs at the fourth passage were differentiated into mesenchymal lineages (adipogenic, osteogenic and chondrogenic differentiation). Original magnification: 40×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

**Fig. 2.** Growth kinetics of MSC culture showing cumulative PDs and passage-specific PDs as a function of time in culture. (A) Cumulative PDs of cells from passage 1 to passages 9–13. (B) PDs of cells harvested at each passage. PDs, population doublings. Values represent mean ± S.E.M. (n = 8). *Significantly different from passage 1 (p < 0.05). For statistical analysis, we conducted a one-way ANOVA followed by a Tukey’s HSD post hoc test.
Fig. 3. (A–C) The analysis of cytokines and growth factors expressed by MSCs. MSCs isolated from six donors were cultured to analyze the expression levels of cytokines and growth factors in the culture media according to serial passages (third, fifth, seventh and ninth). Values represent mean ± S.E.M. (n = 6).*Significantly different from passage 3 (p < 0.05). For statistical analysis, we conducted a one-way ANOVA followed by a Tukey's HSD post hoc test.

Cultured media obtained from the third, fifth, seventh and ninth passages were analyzed. IL-6, VEGF and IL-8, which were reported as cytokines abundantly expressed in MSCs [11,17], were highly secreted and detected at more than 50 pg/10,000 cells at all passages (Fig. 3A). Importantly, the expression levels of these cytokines and growth factors decreased gradually over successive passages (Fig. 3). When compared to third passage MSCs, VEGF, IL-15, GM-CSF, IL-10, PDGF-bb and basic FGF expression levels were decreased at the seventh passage, and all cytokines and growth factors except G-CSF showed a significantly lower level at the ninth passage. Our results indicate that the secretion of cell adhesion molecules, anti-inflammatory cytokines and growth factors by MSCs progressively declines with successive passages.

As a rare and fatal neurodegenerative disease with no effective treatment, ALS is an ideal candidate for stem cell therapy. MSCs express and secrete various cytokines and growth factors that have beneficial effects for a number of central nervous system disease models. However, in vitro expansion and subculturing of MSCs are needed prior to clinical use because of the low density of MSCs in the BM. To date, the various parameters for successive passaging of MSCs have been poorly evaluated.

Previous reports [6,22] have demonstrated that human MSCs are heterogeneous in that they contain at least three morphologically distinct cell types: very small round cells, thin spindle-like cells and large flat cells. Very small round cells and thin spindle-like cells rapidly divide, while large flat cells slowly divide. In our study, the majority of cells in primary culture and early passages were a homogenous population of small spindle-like cells. The number of large, slowly dividing cells increased in the cell population during in vitro expansion and the PDs gradually decreased after the fifth to seventh passages. These observations led to the interpretation that the spindle-like cells are multipotent adult progenitor cells (MAPC) that gradually give rise to large flat cells through asymmetric cell division, resulting in heterogeneity in the cell population.

Digirelamo et al. [7] reported that late-passage MSCs formed fewer adipocytes than early passage MSCs. We determined the differentiation potential of early-passage (fourth) and late-passage (ninth) MSCs. Early passage MSCs differentiated into mesodermal tissue cells (adipocytes, osteocytes or chondrocytes), but late-passage cells failed to differentiate into these cell types, even though the characteristics of antigenic expression of the fourth and ninth passage MSCs were maintained (Supplementary Table 1). The study by Campagnoli et al. [5] is the only report showing maintenance of adipocyte differentiation potential during long-term culture. However, the authors used fetal MSCs, which may differ from adult MSCs. Other researchers have mentioned that morphological changes of cells due to long-term culture lead to telomere shortening, which results in senescence [10,16]. It is therefore possible that the change in cell morphology that occurs during successive passaging can induce the senescence of MSCs, rather than differentiation. A few studies have evaluated the alteration of MSCs in long-term culture.

Given the hypothesis that the secretion of various cytokines and growth factors by MSCs plays important roles in modulating the BM microenvironment and hematopoiesis, some studies have investigated cytokine expression in MSCs [11,17]. The study of Haynesworth et al. [11] detected the expression of various cytokines, including IL-6, G-CSF, SCF and IL-11, in the growth medium of BM-derived MSCs. Liu and Hwang [17] detected several cytokines and growth factors, including GM-CSF, IL-1β, IL-6, IL-8, MCP-1 and VEGF in the cord blood-derived MSCs (CB-MSCs) and demonstrated that IL-1β, a major intermediary of inflammation and immunological reactions [21], regulated the expression of IL-6 in CB-MSCs. In the two studies mentioned above, MSCs originating from the BM and CB secreted high levels of cytokines such as IL-6, VEGF, IL-8, and MCP-1. Of those cytokines, IL-6 plays an important role in the differentiation and regeneration of various stem cells, inhibits osteoblast development and promotes neuronal...
survival and astrocyte proliferation [8, 12, 15]. We tested the expression pattern of cytokines and growth factors secreted from MSCs from the third, fifth, seventh and ninth passages. IL-6, VEGF, IL-8, IL-10, IL-15, GM-CSF, PDGF-bb, G-CSF, basic FGF, IFN-γ and IL-1β were detected, but their levels gradually decreased during long-term culture. Of the cytokines and growth factors tested, IL-6, VEGF and IL-8 were abundantly expressed by MSCs at these four passages. High levels of VEGF can stimulate PDGFRs, thereby regulating MSC migration and proliferation [1]. The gradual decrease in IL-6 and VEGF from the third to ninth passage therefore appears to be related to the reduced growth rate during long-term culture. Further, the decrease in expression of IL-1β and IL-6 during long-term culture supports the previous finding that IL-1β is a regulator of IL-6 [17, 18]. In addition to the cytokines already mentioned, we simultaneously analyzed 16 other cytokines, including IL-9, IL-17, IL-20 (P70), IL-7, MCP-1, MCP-1, IL-4, IL-13, Eotaxin, IL-10, IL-2, RANTES, TNF-α, IL-5, IL-1ra and MIP-1β. The expression of these cytokines also showed a gradual decrease as the passage number increased, with the most significant decrease observed at the seventh and ninth passages.

As a result, long-term culture of MSCs is likely to reduce the differentiation potential and proliferation of MSCs. Therefore, when autologous BM-derived stem cell therapy is applied intractable ALS patients for the purpose of neuroprotection, earlier passages of MSCs might have more beneficial effects in ALS patients. This finding is an important consideration for the clinical application of autologous ALS stem cell therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2010.01.054.

References