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Neuropotency of Human Mesenchymal Stem Cell Cultures: Clonal Studies Reveal the Contribution of Cell Plasticity and Cell Contamination

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ABSTRACT

Various studies have shown neuropotency of bone marrow-derived human mesenchymal stem cells (hMSC) based on the appearance of cells with neural phenotype before or after neural induction protocols. However, to date, it is unclear which mechanisms account for this observation. We hypothesized that neural phenotypes observed in hMSC cultures can be because of both intrinsic cell plasticity and contamination by cells of neural origin. Therefore, we characterized 38 clones from hMSC cultures by assessing their adipogenic/osteogenic potential with specific mesenchymal differentiation protocols, and their molecular neural phenotype by RT-PCR analysis before and after exposure to a defined neural stem cell (NSC) medium for 8 days (neural protocol). We found 33 clones with mesenchymal potential and 15 of them also showed a neural phenotype. As neural phenotypes were maintained during the neural protocol, this suggested neural cell plasticity in 39% of all clones through pluripotency. Importantly, we were able to induce neural phenotypes in 11 of mesenchymal clones applying the neural protocol, demonstrating neural cell plasticity in 29% of all clones through the mechanism of transdifferentiation. Finally, 2 of 5 nonmesenchymal clones (5% of all clones) displayed a neural phenotype indicating neural cell contamination of hMSC cultures. In conclusion, we found 2 different ways of neuropotency of hMSC cultures: cell plasticity and cell contamination.

KEY WORDS

Mesenchymal stem cells • Multipotent stromal cells • Neuropotency • Pluripotency • Transdifferentiation • Neural cell plasticity • Neural cell contamination

INTRODUCTION

Bone marrow contains hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC), also termed multipotent stromal cells, which can be separated in vitro by the property of MSC to adhere to plastic. They can be identified using standard MSC markers, as alpha-smooth muscle actin (ASMA) and surface marker CD105, and the absence of expression of the HSC markers CD45 and CD34 for their molecular characterization [1,2]. However, the "gold standard" to label these cells as MSC is the probe of their ability to differentiate into adipocytes, osteocytes, and chondrocytes [3,4]. On the other hand, various in vitro studies showed neuropotency of human MSC (hMSC) based on their expression of specific neural protein after in vitro stimulation with neural differentiation media [5,6]. In agreement with other definitions, "transdifferentiation" represents the activation of a new differentiation program. Therefore, when researchers show the appearance of neural phenotypes in hMSC cultures after applying neural induction media, they argue that cells are transdifferentiating toward neural lineage [7]. Recently, it has been shown that undifferentiated hMSC cultures isolated by plastic adhesion expressed low levels of neural-specific markers before any differentiation [8]. To date, it is unclear why some undifferentiated hMSC culture-derived cells have neural phenotype. We suggest that this might be because of pluripotency of hMSC culture-derived cells and also the presence of contaminated neural cells in the cultures.

To prove our hypothesis, we established clones from hMSC cultures and characterized them assessing

mesenchymal differentiation potential and the neural gene expression patterns before and after exposure to the neural protocol. Data analysis allowed us to define the clones as mesenchymal versus nonmesenchymal and place them in a hierarchy relative to their molecular neural phenotype considering that neuroD1 expression revealed neural fate [9]; nestin expression, neural stem cell (NSC) phenotype [10]; medium chain neurofilament (NF-M) expression, neuronal phenotype [11] and, GFAP and GalC expression, glial phenotypes [12,13].

Our clonal study demonstrates that the neuropotency of hMSC cultures depends on both mesenchymal and nonmesenchymal subpopulations of cells, including those of neural origin, which are present in bone marrow-derived cultures. Additionally, our neural protocol may serve to efficiently obtain neural-like cells from hMSC cultures.

MATERIAL AND METHODS

Isolation and Expansion of hMSC Cultures

Leftover material was obtained from 4 heparinized aspirates of bone marrow from normal individuals undergoing bone marrow harvests for allogeneic transplantation, as part of a protocol approved by the Ethical Committee of the Hematology Department of Clinica Alemana (Santiago, Chile). Samples were diluted in 1/5 (v/v) phosphate-buffered saline (PBS) and centrifuged at 400 $\times$ *g* for 7 minutes. Total cells were seeded at a density of 1×10^6 nucleated cells/cm^2 in α-10 medium composed by α-minimal essential medium (α-MEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (v/v), 0.8 mg/L gentamicine (Biosano laboratory, Santiago, Chile). Cultures were incubated in a humidified atmosphere at 37°C in 5% CO_2. After 24 hours, nonadherent cells were removed by replacing culture medium. When foci of fibroblastic-like cells were confluent, cells were detached with 0.25% (p/v) trypsin, 2.65 mM EDTA (Gibco), and subcultured at 7×10^3 cells/cm^2 for further expansion. Isolated cells were characterized by real-time RT-PCR for CD34, CD105, and ASMA, together with the evaluation of their capacity to differentiate in vitro into adipocytes, chondrocytes, and osteocytes.

Isolation and Expansion of hMSC Culture-Derived Clones

Clones were obtained from hMSC cultures by limiting dilution. Briefly, 100 cells/well were seeded in the first column of a 96-well plate, and immediately serial 1:2 dilutions were performed to reach 1 cell/well. To ensure single-cell clones, wells were examined daily under a phase-contrast microscope, and those containing more than 1 cell were excluded from the study. Single cells cultured in fresh medium were not able to proliferate. Single cells only grew when cultured with a mixed medium composed of 50% fresh medium and 50% conditioned medium collected from subconfluent hMSC cultures after 48 hours, centrifuged, and filtered through a 0.2 μm filter (Fisher Scientific International Inc., Pittsburgh, PA) to eliminate cellular components. Clones were expanded and further characterized according to their mesenchymal and neural phenotypes.

Mesenchymal Differentiation Protocols

For adipogenic and osteogenic lineages, cells were seeded at a density of 2.5×10^4 cells/cm^2 and later stimulated with adipogenic (α-10 medium supplemented with 100 μg/mL isomethylbutylxanthine [Calbiochem, La Jolla, CA], 1 μM dexamethasone, 0.2 U/mL insulin [Humalog], and 100 μM indomethacin [Sigma-Aldrich, St. Louis, MO], or osteogenic (α-MEM + 10% FBS with 0.1 μM dexamethasone, 50 μg/mL ascorbate-2-phosphate, and 10 mM β-glycerophosphate; Sigma-Aldrich) differentiation media during 10 and 21 days, respectively. Nonstimulated cultures were used as control and maintained in α-10 medium. Media were replaced twice a week. To assess adipogenic and osteogenic differentiation, intracellular lipid droplets were revealed by staining with Oil Red O (Merck, West Point, PA) and matrix mineralization by staining with Alizarin Red (Sigma-Aldrich), respectively [1].

For chondrogenic lineage, cells were cultured at a density of 5×10^3 cells/μL in 10 μL of α-10 medium to achieve the adequate tridimensional conditions for micromass formation. After 2 hours the α-10 medium was replaced with the chondrogenic differentiation medium modified from that used by Pittenger et al. [3]. After 7 days, the proteoglycans that compose the extracellular matrix of the micromass were revealed by staining with Safranin O (Merck).

Neural Protocol

Cells were seeded in α-10 medium at a density of 4-6 $\times$ 10^3 cells/cm^2. After 24 hours, the medium was replaced with NSC medium similar to that used in human NSC cultures [14], containing DMEM/F12 (1:1) (Gibco), 1% bovine serum albumin (BSA, Merck), 6 g/L D(+)-glucose (Merck), 0.8 mg/L gentamicin, supplemented with N2 and B27 supplements (Gibco), human epidermic growth factor (EGF), and human basic fibroblast growth factor (bFGF), both at 10 ng/mL (R&D Systems, Minnespolis, MN), in which the cells were cultured until the end of the experiment. Neural phenotype was evaluated according to the pattern of expression of neural genes, before (0 days) and after (8 days) exposure to NSC medium.

Isolation and Characterization of Rat NSC (rNSC) from Olfactory Bulbs

Two olfactory bulbs were isolated from each postnatal (P0-P4) rat. Disaggregated cells were plated in suspension at cell density of 6×10^5 cells/mL in