Characterization of Autologous Mesenchymal Stem Cell-Derived Neural Progenitors as a Feasible Source of Stem Cells for Central Nervous System Applications in Multiple Sclerosis

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Key Words. Autoimmune disease • Autologous stem cell transplantation • Mesenchymal stem cells • Progenitor cells

ABSTRACT

Bone marrow mesenchymal stem cell-derived neural progenitors (MSC-NPs) are a potential therapeutic source of cells that have been shown to be efficacious in a preclinical model of multiple sclerosis (MS). To examine the feasibility of using MSC-NPs as an autologous source of cells to promote central nervous system (CNS) repair in MS, this study characterized human MSC-NPs from a panel of both MS and non-MS donors. Expanded MSCs showed similar characteristics in terms of growth and cell surface phenotype, regardless of the donor disease status. MSC-NPs derived from all MSCs showed a consistent pattern of gene expression changes that correlated with neural commitment and increased homogeneity. Furthermore, the reduced expression of mesodermal markers and reduced capacity for adipogenic or osteogenic differentiation in MSC-NPs compared with MSCs suggested that MSC-NPs have reduced potential of unwanted mesodermal differentiation upon CNS transplantation. The immunoregulatory function of MSC-NPs was similar to that of MSCs in their ability to suppress T-cell proliferation and to promote expansion of FoxP3-positive T regulatory cells in vitro. In addition, MSC-NPs promoted oligodendroglial differentiation from brain-derived neural stem cells that correlated with the secretion of bioactive factors. Our results provide a set of identity characteristics for autologous MSC-NPs and suggest that the in vitro immunoregulatory and trophic properties of these cells may have therapeutic value in the treatment of MS.

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) associated with a progressive clinical course and significant physical or cognitive disability [1]. Demyelinated MS lesions in the brain and spinal cord are characterized by a combination of immune-cell infiltration, oligodendrocyte loss, failure of oligodendrocyte precursor cells to remyelinate, axonal pathology and degeneration, and ultimately astrogliosis and scar formation. Given the aberrant immune response underlying MS pathogenesis, current disease-modifying treatments for MS consist of immunomodulatory therapies. However, these treatments are only partially effective in slowing down the progressive phase of MS, which may be largely neurodegenerative and/or may involve autonomous abnormalities in immune function that are not as amenable to this type of therapy. Thus, there is an urgent need for therapies that can stop or reverse the progression of MS through strategies that target degenerative disease processes within the CNS. In particular, the regenerative potential of cell-based biological therapies is an active area of investigation for the treatment of MS.

The development of cell-based repair strategies for MS faces the challenge of delivery to multifocal lesions throughout the brain and spinal cord. Given this pathology, a viable strategy for CNS dissemination of therapeutic stem cells is to deliver them into the cerebrospinal fluid. Further challenges include compatibility with the CNS environment in order to avoid ectopic tissue formation or immune activation, which might exacerbate the already inflammatory situation in MS. For these reasons, an initial strategy is to harness the CNS regenerative potential of an autologous source of cells by intrathecal delivery.

Mesenchymal stem cells (MSCs) are an obvious source of stem cells for MS because they are readily expanded from adult bone marrow and because they can be used autologously. The therapeutic potential of MSCs has been attributed to their paracrine effects on neighboring host cells, leading to immunomodulatory,
trophic, antiapoptotic, angiogenic, neuroprotective, and antioxidant effects [2]. The potent immunoregulatory properties of MSCs on both innate and adaptive immune responses have led to preclinical testing of MSCs targeting autoimmune dysregulation in MS. Intravenous delivery of MSCs into the experimental autoimmune encephalomyelitis (EAE) animal model of MS resulted in induction of peripheral T-cell tolerance, promotion of Th2 immune responses, and amelioration of inflammatory onset and severity of disease [3–7]. Appropriately, clinical trials using intravenous autologous MSCs in inflammatory forms of MS have commenced [8–10]. Results from an initial 10-patient study of intravenous MSCs demonstrated modest visual improvement in some patients with secondary progressive MS [11].

MSCs exhibit a certain degree of differentiation plasticity and are capable of mesodermal differentiation into skeletal, chondrogenic, and adipogenic lineages, as well as neuroectodermal differentiation into a neural progenitor-like cell population referred to as MSC-derived neural progenitors (MSC-NPs) [12, 13]. MSC-NPs exhibit “neurosphere” morphology, upregulation of neural-specific genes, and in vitro electrophysiological properties indicating functional neuronal differentiation [12–16]. Neuroectodermally converted MSC-NPs showed decreased capacity for adipogenic or osteogenic differentiation in vitro [15], suggesting that this cell population may be more appropriate for clinical application in the CNS by limiting the potential risk of uncontrolled mesodermal lineage differentiation or formation of other ectopic tissue [17, 18]. To test the preclinical efficacy of MSC-NPs in MS, we recently showed that intrathecal injections of mouse-derived MSC-NPs during chronic EAE resulted in a significant reduction of chronic disability through focal immunomodulatory and trophic mechanisms, which was correlated with a reduced area of demyelination [15].

The anticipated autologous use of MSC-NPs for CNS repair in MS warrants characterization of human MSC-NPs and confirmation that MSC-NPs derived from MS patients do not significantly differ from those of healthy donors. We examined MSC-NPs from 10 patients with progressive MS and compared them with MSCs and with four control donors. We found characteristic up-regulation of neural-specific markers and reduced capacity for mesodermal differentiation in MSC-NPs from both MS and healthy donors. MSC-NPs display in vitro immunoregulatory and trophic properties, suggesting a therapeutic mechanism of action in MS. This study provides a critical first step to the development of MSC-NPs for future therapeutic use.

**Materials and Methods**

**Cell Culture**

Primary MSCs were harvested from bone marrow aspirates (10 ml) from patients seen at the International MS Management Practice (New York) with institutional review board approval (St. Luke’s Roosevelt Hospital Center Institutional Review Board, New York) and informed consent (supplemental online Table 1). Two healthy control bone marrow aspirates were obtained commercially (Lonza, Walkersville, MD, http://www.lonza.com). Mononucleated cells were isolated by density gradient centrifugation with 1.073 g/ml Ficoll-Paque Premium (GE Healthcare Life Sciences, Pittsburgh, http://www.ge lifesciences.com), and MSCs were further isolated based on plastic adherence under low-oxygen (5%) conditions. Control MSCs were expanded in MSC growth medium (MSCGM) (Lonza) containing 10% fetal bovine serum (FBS). MS-MSCs were isolated and expanded in 10% autologous serum (AS). The medium was changed every 3–4 days. The cells were passaged at 80% confluence with TrypLE (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and replated at a density of 2,000 cells per cm². 7071L MSCs were obtained from the Center for the Preparation and Distribution of Adult Stem Cells, Texas A&M Health Sciences Center, and expanded as described above. Population doublings were determined by log N/log2, where N = number of cells plated per number of cells harvested. All of the MSCs used for experimentation were between passages 3 and 8.

For MSC-NPs, MSCs were cultured in low-adherence flasks in serum-free neural progenitor maintenance medium (NPMM) (Lonza) containing 20 ng/ml each of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) for 21 days with a medium change every 2–3 days. Floating “neurospheres” were visible after 2–5 days. All of the MSC-NPs used in this study were obtained after 21 days in NPMM and were not passaged further. To obtain single-cell suspensions of MSC-NPs for experimentation, neurospheres were triturated in TrypLE 20 times with a fire-polished glass pipette before plating. Trypan blue staining and hemacytometer counting were performed to confirm single-cell suspension and >80% viability. Cytogenetic analysis by G banding was performed by Clinical Laboratory Services at Columbia University Medical Center (New York) on MSCs expanded between passages 3 and 7.

**Flow Cytometry**

Cell surface staining was carried out using fluorescein isothiocyanate- or phosphatidylethanolamine-conjugated mouse antibodies against human CD90, CD73, CD34, CD14, CD19, HLA-DR (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com), and CD105 (eBioscience, San Diego, www.ebioscience.com) and compared with appropriate isotype controls. Cell surface CXCR4 staining was carried out using rabbit anti-CXCR4 primary antibody 1:100 (Chemicon, Temecula, CA, http://www.chemicon.com) or isotype control followed by secondary antibody staining with anti-rabbit IgG conjugated to Alexa 488 (1:2,500). For intracellular antigen staining, the cells were fixed, permeabilized, and then incubated with rabbit anti-Nestin 1:5,000 (Chemicon), rabbit anti-gliarial fibrillary acidic protein (GFAP) 1:500 (Dako, Glostrup, Denmark, http://www.dako.com), rabbit anti-Neurofilament-M (NF-M) 1:1,000 (Chemicon), mouse anti-α-actin smooth muscle isoform (SMA) 1:1,000 (Chemicon), or the appropriate unconjugated isotype controls. Secondary antibodies were Alexa 488-conjugated anti-rabbit or anti-mouse IgG. Analysis was performed on a FACSARia flow cytometer (BD Biosciences). Mean fluorescence intensity (MFI) was determined for each histogram, and fold increase in MFI for each antibody over its isotype control was determined for each cell population.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from MSC and MSC-NP paired samples using RNeasy Plus (Qiagen, Hilden, Germany, http://www.qiagen.com), and first-strand cDNA was synthesized from equal amounts of RNA from each sample using Superscript III (Invitrogen). Quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed using TaqMan Universal PCR Master Mix and prevalidated TaqMan gene expression assays (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) to detect human...
SMA (ACTA2-Hs00426835_g1), CD90 (THY1-Hs00174816_m1), GFAP (Hs00090928_g1), NF-M (Hs01935727_m1), Nestin (Hs00701210_s1), CXCR4 (Hs00607978_s1), indoleamine-2,3-dioxygenase (IDO) (Hs00158032_m1), transforming growth factor β (TGFβ) (Hs99999918_m1), Toll-like receptor 2 (TLR2) (Hs01872448_s1), TLR3 (Hs00152933_m1), TLR4 (Hs00152939_m1), interleukin-10 (IL-10) (Hs00174086_m1), CXCL10 (Hs00171042_m1), IL-6 (Hs00174131_m1), glial-derived neurotrophic factor (GDNF) (Hs01931883_s1), hepatocyte growth factor (HGF) (Hs00300159_m1), insulin-like growth factor (IGF) (Hs01547656_m1), IL-11 (Hs00174148_m1), vascular endothelial growth factor (VEGF) (Hs00900055_m1), brain-derived neurotrophic factor (BDNF) (Hs00601650_m1), and bFGF (Hs00960934_m1) gene expression along with 185 (Hs99999901_s1) endogenous control gene. Q-RT-PCR was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems), and relative quantification was determined using RQ Manager software (Applied Biosystems).

**Immunocytochemistry**

For immunocytochemistry, MSCs and MSC-NPs were plated on eight-well chamber slides in MSCGM, fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100, and blocked with 5% goat serum. The cells were incubated overnight 4°C with rabbit anti-Nestin 1:2,000 (Chemicon), rabbit anti-GFAP 1:500 (Dako), rabbit anti-NF-M 1:1,000 (Chemicon), mouse anti-SMA 1:1,000 (Chemicon), rabbit anti-CXCR4 1:100 (Chemicon), mouse anti-Cd90 1:1,000 (BD Biosciences), or the appropriate unconjugated isotype controls. For coculture assays, fixed cells were stained with anti-galactocerebroside (GaIC) 1:200 (Chemicon), anti-b-3-tubulin (TuJ1) 1:500 (Chemicon), anti-GFAP 1:200 (Dako), and anti-myelin basic protein (MBP) 1:500 (Covance, Princeton, NJ, http://www.covance.com). Triton was omitted for GaIC staining. Human nuclei were detected by anti-human nuclear antigen (hNA) 1:100 (Chemicon). Primary antibodies were detected with either anti-rabbit or isotype-specific anti-mouse secondary antibodies (1:1,000) conjugated with Alexa 488 or 594 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). 4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen) was used for detection of all cell nuclei. Negative control samples were performed omitting the primary antibody. Fluorescent images were captured at a magnification of ×20 with an LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

**Adipogenic and Osteogenic Differentiation**

MSCs or MSC-NPs were plated at 30,000 cells per cm² in MSCGM until confluence was reached. For adipogenic differentiation, the cells were incubated for 17 days alternating between 3–4-day cycles of induction medium (MSCGM containing 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin, and 100 μM indomethacin) and 1–2 days with maintenance medium (MSCGM containing 10 μg/ml insulin). Control cells contained maintenance medium only. To visualize lipid accumulation, the cultures were fixed in 10% formalin, stained with 0.3% Oil Red O in 60% isopropanol, and visualized by light microscopy.

For osteogenic differentiation, the cells were plated as described above and cultured in osteogenic induction medium (MSCGM containing 0.1 μM dexamethasone, 0.2 mM ascorbic acid, and 10 mM glycerol 2-phosphate). Control wells contained MSCGM alone. After 17 days, the cells were fixed in 10% formalin, stained with 1% alizarin red S, and visualized by microscopy. For quantification of calcium deposition, the cells were extracted in 0.5 N HCl overnight and briefly centrifuged, and the calcium content of the supernatant was determined by a CPC Liquicolor kit (Stanbio Laboratory, Boerne, TX, http://www.stanbio.com). For gene expression analysis, Q-RT-PCR was performed for adiponectin (ADIPQ) (Hs00605917_m1), peroxisome proliferator-activated receptor γ (PPARγ) (Hs01115513_m1), alkaline phosphatase (ALPL) (Hs01029144_m1), and integrin-binding sialoprotein (IBSP) (Hs00173720_m1) (Applied Biosystems). The values were compared with the average expression in MSCs after adipogenic or osteogenic differentiation.

**Immune Cell Coculture Assays**

MSCs or MSC-NPs were plated 10,000 cells per cm² in 24-well plates in duplicate and allowed to adhere for 24 hours. Allogeneic peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor volunteers and labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE). PBMCs were plated at a 10:1 ratio with MSCs or MSC-NPs in RPMI medium with 10% FBS. The cells were stimulated with 2 μg/ml phytohemagglutinin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) for 72 hours and then stained with anti-human CD4-eFlour 450 (eBioscience) or with isotype control. Proliferation was measured by decreased CFSE intensity by flow cytometry. For regulatory T-cell analysis, MSCs or MSC-NPs were cocultured as above with purified CD4+ T cells isolated from allogeneic PBMCs using a naive CD4+ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). After 72 hours of stimulation with anti-CD3/CD28 Dynabeads (Invitrogen), the cells were harvested and stained for CD4 as above, followed by intracellular staining using anti-human FoxP3-APC (eBioscience) or isotype control. Total RNA was isolated from parallel wells, and FoxP3 mRNA levels were determined by Q-RT-PCR as described above using FoxP3-specific primer set (Hs01085832_m1). Conditioned medium from coculture experiments was collected and stored at −20°C until use. The levels of human tumor necrosis factor α (TNFα), IL-12 (p70), interferon γ (IFNγ), and IL-6 were measured by Bio-plex Pro 4-plex assay and read on a Bio-Plex 200 reader (Bio-Rad, Hercules, CA, http://www.bio-req.com). TGFβ was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). The data are expressed relative to the control (without coculture).

**Trophic Coculture Assays**

For trophic coculture experiments, MSC-NPs were plated at 10,000 cells per cm² on laminin/poly-o-lysine-coated eight-well slides (BD Biosciences). Rat neural stem/progenitor cells (rNSCs) (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) were cultured in NeuroCult NS-A rat proliferation medium containing EGF, bFGF, and 0.2% heparin, and the cells were expanded according to the manufacturer’s instructions. Dissociated rNSCs were plated at a 1:1 ratio with or without MSC-NPs, and differentiation was induced by growth factor withdrawal using NeuroCult NS-A rat differentiation medium for 14 days. Immunocytochemistry was performed as described above. Primary antibodies used in coculture assays were not species-specific. Quantification was performed manually by counting GaIC-, GFAP-, or b-3-tubulin-positive cells in a
minimum of six representative fields per well. The number of positive cells was divided by the total number of DAPI-positive/hNA-negative cells per well.

RNA was purified from duplicate wells in parallel to immunofluorescence, and Q-RT-PCR was performed in duplicate as detailed above using primer sets for proteolipid protein (PLP) (Mm00456892_m1), GFAP (Gfap-Mm00546086_m1), and β3-tubulin (Mm00727586_s1). All primers cross-react with rat genes.

Cell culture supernatants were analyzed for stromal cell-derived factor 1 (SDF1), VEGF, HGF, and bFGF using the Human Circulating Cancer Biomarker Magnetic Bead Panel 1 (Millipore, Billerica, MA, http://www.millipore.com) Luminex assay. BDNF and GDNF were analyzed using multiplexed polystyrene bead kits, and TGF-β1 was analyzed by multispecies singleplex bead kit (Invitrogen). IGF-1 and IL-11 levels were determined by ELISA (R&D Systems).

Statistical Analysis

The data were analyzed by one-way analysis of variance to test differences between multiple groups, followed by Newman-Keuls multiple comparison post test or unpaired Student’s t test to determine significance between two groups. Q-RT-PCR results were analyzed by paired Student’s t test. Prism 4 (GraphPad Software, Inc., San Diego, http://www.graphpad.com) was used to calculate significance. Statistical significance was set to p values < .05.

RESULTS

MSCs Derived from MS Patients Are Phenotypically Normal

We initially characterized MSCs from MS patients in order to confirm that MS-MSCs used as a source for MSC-NPs were similar to those of healthy controls. MSC characterization was based on the minimal criteria for defining multipotent MSCs set by the International Society for Cellular Therapy [19]. Both control MSCs and MS-MSCs (supplemental online Table 1) displayed similar growth kinetics (Fig. 1A) and characteristic MSC morphology (Fig. 2A). Both control MSCs and MS-MSCs showed characteristic surface antigen expression (Fig. 1C, 1D) [19]. MS-MSCs were also comparable to control MSCs in terms of multipotential differentiation into adipogenic and osteogenic lineages when assayed by lineage-specific staining and by Q-RT-PCR (data not shown), confirming a previous report [20]. These data confirm that MS-MSCs show similar growth and phenotypic characteristics compared with MSCs from healthy or non-MS donors [20, 21].

Because of the regulatory issues associated with the use of FBS for the expansion of MSCs for clinical use, all of the MS-MSCs in this study were derived in media containing AS. We tested for any differences in MSC growth and phenotype when MS-MSCs were switched to FBS-containing media.
There were no significant differences in MS-MSC proliferation (Fig. 1B) or cell surface antigen expression (data not shown) when cultured in FBS compared with AS.

**Chromosomal Analysis of MSCs**

The potential for clinical use of autologous MSCs warrants confirmation of genomic stability to prevent unwanted malignant transformation of MSCs after transplantation. We therefore karyotyped two control and nine MS patient-derived MSC lines. One of the MSC lines from an MS patient (MS-052) displayed a structural rearrangement between the long arms of chromosomes 3 and 7 in 35% of the cells analyzed at passage 3. Repeated cytogenetic analysis of the MS-052 MSC line demonstrated the same chromosomal translocation after passage 5, again in 35% of cells. Repeat bone marrow aspiration and MSC isolation from the same donor resulted in expanded MSCs with a normal karyotype, suggesting that the original translocation occurred during ex vivo expansion.

**Neural Characteristics of MSC-NPs**

MSC-NPs were derived from MSCs by culture selection in neural progenitor-specific medium for 3 weeks as previously described [12, 15, 16]. MSC-NPs formed cell clusters and grew in suspension (Fig. 2B), exhibiting the hallmark neurosphere morphology of CNS-derived neural stem cells, where the majority of the cells within the neurosphere express Nestin (Fig. 2B, inset) as well as other neural stem cell markers such as musashi-1 [14]. The self-renewal capacity of MSC-NPs was previously verified by passing MSC-NPs for an additional two to five passages [12, 14]. To characterize the rate of neural conversion of MSC-NPs within the first 21 days of culture in neural progenitor medium, we examined the time course of gene expression differences in paired MSC-NPs from both healthy controls and from MS patients (Fig. 2D). Changes in gene expression were sustained for the duration of the 21-day culture period. This trend in gene expression was consistent in MSC-NP/MSC pairs from both healthy controls and from MS patients (Fig. 2D). Changes in gene expression were also seen at the protein level, as shown by immunocytochemistry and flow cytometry (Fig. 3A, 3B). These changes are consistent with previously published studies.

**Figure 2.** Characterization of MSC-NP morphology and gene expression. (A): Spindle-shaped morphology of human MSCs viewed by light microscopy at a magnification of ×10. (B): Spherical morphology of MSC-NPs after 3 weeks of neural induction. The cells were viewed by light microscopy at a magnification of ×20. Inset: Nestin immunofluorescence (red) of MSC-NP neurosphere showing that the majority of 4',6-diamidino-2-phenylindole-positive cells (blue) expressed Nestin. The cells were viewed by fluorescence microscopy at a magnification of ×20. (C): Time course of gene expression changes upon MSC-NP induction. MSC-NPs, compared with MSCs, showed increased mRNA expression of neural markers Nestin, NF-M, GFAP, and CXCR4 and decreased expression of MSC markers CD90 and SMA. The values represent the means ± SE of four separate MSC donors. (D): Panel of gene expression changes in MSC-NPs derived from control (n = 4) or MS (n = 10) patients. Abbreviations: GFAP, glial fibrillary acidic protein; MS, multiple sclerosis; MSC, mesenchymal stem cell; MSC-NP, mesenchymal stem cell-derived neural progenitor; NF-M, Neurofilament-M; SMA, α-actin smooth muscle isoform.
showing increased Nestin expression in MSC-NPs [12, 14–16, 22]. The consistency and specificity of these gene expression changes allow for a quantitative assessment of the cell differentiation status necessary for identity testing of each lot of autologous MSC-NPs and highlight the differences between MSC and MSC-NP cell populations.

MSC-NPs Display Limited Osteogenic and Adipogenic Differentiation Capacity

A defining characteristic of multipotential MSCs is their ability to undergo in vitro differentiation into adipocytes and osteocytes [19]. Previous studies showed that the homologous mouse population of MSC-NPs lacked mesodermal differentiation capability [15]. We therefore tested whether human MSC-NPs could differentiate along adipogenic or osteogenic lineages. Compared with MSCs, MSC-NPs displayed a limited ability to undergo osteogenic differentiation as evidenced by decreased alizarin red staining (Fig. 4A) and significantly decreased calcium deposition (Fig. 4C). Gene expression analysis of MSCs and MSC-NPs after osteogenic differentiation demonstrated decreased expression of ALPL and IBSP (Fig. 4D), which are both downstream effectors of Runx2 and are increased during osteoblast maturation and mineralization of bone [23]. Similarly, when cultured under adipogenic differentiation conditions, MSC-NPs formed far fewer Oil Red O lipid vacuoles (Fig. 4B) and expressed decreased levels of ADIPOQ and PPARγ (Fig. 4E) compared with MSCs. ADIPOQ controls fat metabolism and is expressed exclusively by adipocytes, and PPARγ is a key regulator of fat metabolism, indicating lack of adipocyte function in differentiated MSC-NPs. A lack of adipogenic/osteogenic differentiation was observed with MSC-NPs from both MS and control patients when assayed by lineage-specific staining as described above (data not shown). These results confirm that MSC-NPs lose the differentiation plasticity associated with MSCs, thus minimizing the potential risk of spurious differentiation into mesodermal tissue upon CNS transplantation.

MSC-NPs Exhibit Immunoregulatory Properties

MSCs suppress T, B, NK, and dendritic cell activation and proliferation and can promote development of FoxP3+ regulatory T cells [2, 24–27]. We investigated whether MSC-NPs retain the immunomodulatory properties of MSCs. In a coculture assay with allogeneic lymphocytes, both MSCs and MSC-NPs, derived from both control and MS donors, suppressed T-cell proliferation (Fig. 5A, 5B) and promoted naïve CD4+ T-cell polarization into FoxP3+ T cells (Fig. 5C), which correlated with increased FoxP3 mRNA expression (Fig. 5D). Cytokine measurements from conditioned media of coculture experiments demonstrated a significant decrease in proinflammatory cytokines TNFα and IL-12, with no difference in IFNγ (Fig. 5E). In addition, the levels of IL-6 were significantly increased in cocultures, and TGFβ levels were slightly increased compared with T cells alone, suggesting that these cytokines might mediate some of the effects on regulatory T cells (Fig. 5E). Both MSCs and MSC-NPs were confirmed to secrete IL-6 and TGFβ (data not shown) [2].
MSC-NPs Promote Neuronal and Oligodendroglial Differentiation from CNS Progenitors

MSCs have been shown to exhibit trophic effects that influence neural stem cell fate in vitro through the secretion of bioactive factors [28, 29]. We therefore investigated the potential trophic effects of MSC-NPs when cocultured with rNSCs. When rNSCs were cultured under differentiating conditions of growth factor withdrawal for 2 weeks, most cells formed astrocytes (GFAP⁺) (Fig. 6C), with limited differentiation into oligodendrocytes (GalC⁺ and MBP⁺) (Fig. 6A, 6B) and neurons (β3-tubulin⁺) (Fig. 6D). Coculture with MSC-NPs resulted in a significant increase in the number of GalC⁺ (Fig. 6E) and MBP⁺ (Fig. 6F) oligodendrocytes, with no apparent effect on the number of astrocytes or neurons (Fig. 6G, 6H).

Quantification of immunocytochemistry confirmed this trend (Fig. 6I), showing a significant increase in the number of oligodendrocytes and only a slight increase in the number of astrocytes. The number of neurons remained unchanged. Similarly, quantitative PCR demonstrated a significant increase in the oligodendrocyte PLP, compared with much smaller effects on astrocytes or neurons as detected by GFAP and β3-tubulin transcript, respectively (Fig. 6J). MSC-NPs derived from both control and MS donors were capable of promoting oligodendroglial differentiation (Fig. 6I, 6J). These results confirm that MSC-NPs, similar to MSCs, can regulate neural stem cell fate in vitro, which may have clinical implications in enhancing neural repair and regeneration in vivo. To rule out any influence of coculture conditions on the differentiation status of MSC-NPs, we included immunofluorescent staining for hNA, a marker for human-derived cells. We did not observe any colocalization between hNA-positive and GalC-, MBP-, or β3-tubulin-positive cells in cocultures (Fig. 6E–6H) or in MSC-NPs cultured alone (data not shown), confirming that the differentiated cells in coculture experiments were indeed rNSC-derived and not MSC-NP-derived. In addition, bone fide rNSC-derived astrocytes (Fig. 6C, 6G) displayed a dramatically higher intensity of GFAP immunostaining compared with MSC-NPs (Fig. 3A), suggesting that the observed astrocyte differentiation occurred in rNSCs but not in MSC-NPs. Finally, all primers used for quantitative PCR were rodent-specific and did not amplify message from MSC-NPs alone (data not shown). Thus, MSC-NPs do not appear to be capable of terminal glial or neuronal differentiation under these in vitro conditions.

To better understand the trophic mechanisms of MSC-NPs, we assayed coculture conditioned medium for the presence of growth factors known to affect neural stem cell differentiation and compared them with cultures of MSC-NPs alone and rNSCs alone. In all conditions, BDNF, bFGF, GDNF, and IL-11 were present only at very low levels and thus are unlikely to contribute significantly to neural stem cell differentiation (Fig. 6K). In contrast, HGF and SDF1 were secreted at significant levels by MSC-NPs, both alone or when cocultured with rNSCs. Neither HGF nor SDF1 were detected in rNSCs alone, suggesting that the source of these factors in the cocultures was MSC-NPs. IGF-1, TGFβ, and VEGF were secreted by both MSC-NPs and rNSCs. In the case of IGF-1 and VEGF, levels in the cocultures were additive, raising the possibility that increased levels of these factors may promote oligodendroglial differentiation of rNSCs. TGFβ levels remained the same with or without coculture and thus did not correlate with differences in rNSC differentiation.
Control non-multiple sclerosis; IFN

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; cont, control, non-multiple sclerosis; IFN, interferon γ; IL, interleukin; MFI, mean fluorescence intensity; MS, multiple sclerosis; MSC, mesenchymal stem cell; MSC-NP, mesenchymal stem cell-derived neural progenitor; TC, T cell; TGF, transforming growth factor β; TNFa, tumor necrosis factor α.

Figure 5. Immunoregulatory properties of MSC-NPs and MSCs are similar. (A): Proliferation of phytohemagglutinin-stimulated CFSE-labeled CD4⁺ T cells (white histogram) is reduced in the presence of cocultured MSC-NPs (black filled histogram) as indicated by a right shift in CFSE fluorescence. (B): Reduced proliferation shown in (A) as measured by increased MFI of CFSE in cocultured T cells (TC + MSC-NP or TC + MSC) compared with T cells alone (TC). MSCs or MSC-NPs were derived from cont or MS donors as shown. (C, D): Coculture of CD3/CD28-stimulated naïve CD4⁺ T cells with MSC-NPs or MSCs promotes FoxP3⁺ T regulatory cells measured by flow cytometry (C) and by FoxP3 expression by quantitative polymerase chain reaction (D). (E): Levels of immunoregulatory factors (TNFa, IL-12, IFNγ, TGFβ, and IL-6) secreted in cocultures from (C) and determined by Luminex assay. MSCs and MSC-NPs in (D, E) were derived from MS donors. The values are shown relative to TC cells alone. In all panels, the values represent the averages of at least two independent experiments, and the error bars represent SD. *, p < .05; **, p < .01, demonstrating significance compared with TC control. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; cont, control, non-multiple sclerosis; IFNγ, interferon γ; IL, interleukin; MFI, mean fluorescence intensity; MS, multiple sclerosis; MSC, mesenchymal stem cell; MSC-NP, mesenchymal stem cell-derived neural progenitor; TC, T cell; TGFβ, transforming growth factor β; TNFa, tumor necrosis factor α.

Upregulation of Candidate Bioactive Factors in MSC-NPs Compared with MSCs

The mechanism(s) mediating either the trophic or the immunoregulatory properties of MSC-NPs is currently unknown, but it most likely involves the secretion of chemoattractants, cytokines, and growth factors, similar to those secreted by MSCs [2, 30]. To compare the expression of candidate trophic growth factors in MSC-NPs to the level expressed in MSCs, we examined their expression by Q-RT-PCR. HGF and IGF were upregulated >10-fold in MSC-NPs compared with MSCs (Fig. 7A), consistent with the high secretion of these factors in MSC-NP cultures. In addition, expression of IL-11, and VEGF were also upregulated between 2- and 10-fold in MSC-NPs compared with MSCs. In contrast, levels of GDNF, SDF1, and TGFβ were similar in MSCs and MSC-NPs, and bFGF mRNA was downregulated MSC-NPs (Fig. 7A). BDNF was undetectable in most samples tested. There was no significant difference in fold change in gene expression between MSC-NPs derived from control or MS donors.

We also tested the expression of candidate cytokines and other immunoregulatory molecules (Fig. 7B). MSC-NPs showed >100-fold upregulation of IDO1, IL-10, and TLR2 transcripts compared with MSCs, whereas CXCL10 expression was upregulated approximately 30-fold. IL-6, as well as other TLRs (TLR3 and TLR4), were expressed at equal levels in both MSCs and MSC-NPs. There were no significant differences in fold change in gene expression between MSC-NPs derived from control and MS donors. Although all of the candidate factors screened are known to be important in MSC-mediated immunoregulatory function [2], the significance of their upregulation in MSC-NPs remains unclear. Whether or not the changes in trophic and immunoregulatory gene expression translate into increased potency of MSC-NPs remains undetermined. Nevertheless, these experiments identify candidate growth factors and cytokines that may play a role in both the immunoregulatory and trophic properties of MSC-NPs.

**DISCUSSION**

The aim of this study was to examine the feasibility of using autologous bone marrow-derived MSC-NPs as a source of stem cells to promote repair and regeneration in MS. Although previous studies have characterized MSCs from MS patients [20, 21], this is the first study to examine the characteristics of MSC-NPs from a panel of bone marrow donors both with and without MS. Our results show that MSC-NPs exhibit a number of properties that support their therapeutic potential in the CNS, including (a) MSC-NPs are neuroectodermally committed and may be more suitable for administration into the CNS; (b) MSC-NPs have reduced capacity for mesodermal differentiation, thus reducing potential risk of ectopic mesodermal differentiation in the CNS; (c) MSC-NPs have immunomodulatory and trophic properties hypothesized to promote repair during CNS damage; and (d) MSC-NPs express and secrete bioactive factors that can mediate repair in various models of CNS injury [31]. MSC-NPs derived from MS patients and healthy controls uniformly displayed these characteristics, with MSC-NPs demonstrating consistent changes in gene expression compared with the MSCs from which they were derived. The identification of MSC-NP-specific markers related to their differentiation status, homogeneity, and biological activity is critical to the development of identity testing of autologous MSC-NPs for future therapeutic use.

Current clinical experience with MSCs is based on their ability to modulate a wide range of immune cell responses. In particular, MSCs can influence T-cell responses by inhibition of T-cell proliferation, promotion of T-cell anergy, suppression of cytotoxic T-cell function, and promotion of a regulatory T-cell phenotype [2]. The mechanisms by which MSCs exert their immunomodulatory effects include the secretion cytokines and factors,

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Figure 6. Trophic effects of MSC-NPs on the oligodendroglial differentiation of rat brain-derived neural stem cells. (A–H): Immunocytochemical analysis of rNSCs alone (A–D) or cocultured with human MSC-NPs (E–H) after 2 weeks of differentiation. Oligodendrocytes were detected by GalC (A, E) (green) and MBP (B, F) (green), astrocytes by GFAP (C, G), and neurons by β3-tub (D, H) (green). Human MSC-NPs in (E–H) were detected by staining for human nuclear antigen (hNA; red). All nuclei were stained by DAPI (blue). One representative experiment is shown using cells derived from a multiple sclerosis (MS) donor. (I): Quantification of immunostaining compiled from two separate experiments using MSC-NPs derived from control and MS donors as shown. Quantification represents positive staining normalized for the number of DAPI-positive, hNA-negative cells. (J): Quantitative polymerase chain reaction of PLP, GFAP, and β3-tubulin mRNA expressed in MSC-NP/rNSC cocultures relative to rNSCs alone. (K): Levels of secreted factors in rNSC/MSC-NP cocultures, or rNSCs or MSC-NPs alone after culturing for 2 weeks under differentiating conditions. Growth factors were measured by Luminex and enzyme-linked immunosorbent assay. (I, J): The values represent the averages of three separate experiments, and the error bars represent SD. *, p < .05; **, p < .01, demonstrating significance compared with rNSC control. Abbreviations: β3-tub, β3-tubulin; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; cont, control; DAPI, 4′,6-diamidino-2-phenylindole; GalC, galactocerebroside; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; IGF1, insulin-like growth factor 1; IL-11, interleukin 11; MBP, myelin basic protein; MS, multiple sclerosis; MSC, mesenchymal stem cell; MSC-NP, mesenchymal stem cell-derived neural progenitor; PLP, proteolipid protein; rNSC, rat neural stem/progenitor cell; SDF1, stromal cell-derived factor 1; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor.
changes translate into similar increases in protein expression, cytokine secretion, and range of immunomodulatory effects remains to be determined. Nevertheless, these findings suggest that MSC-NPs may exert a similar if not a more potent capacity to influence inflammatory responses compared with MSCs through upregulation of these specific cytokines and factors. MSC-NPs may suppress an immune response present in the CNS at the time of injection and may actively promote an environment conducive to repair and regeneration.

Also pertinent to MSC-based therapies targeted toward CNS repair are the “bystander” mechanisms whereby MSCs exert local effects on damaged tissues through the secretion of bioactive factors [31]. These trophic effects of MSCs include neuroprotective effects, as well as promotion of neurogenesis and oligodendrocyte differentiation of neural stem cells. Analysis of candidate trophic factors revealed that levels of HGF, IGF-1, SDF1α, and VEGF were all associated with an increase in the number of oligodendrocytes in the coculture, and all of these factors have been shown to play a role in oligodendrocyte differentiation [39–42]. Interestingly, MSC-NPs showed upregulated mRNA expression of these factors compared with MSCs, suggesting potential differences between MSCs and MSC-NPs in the mechanisms or potency of these trophic effects. The trophic properties of MSC-NPs characterized in vitro correlate with our previous findings in vivo demonstrating an increase in endogenous neural stem cell populations upon intrathecal MSC-NP injections in EAE mice [15]. Future studies will be aimed at the identification of the secreted factor(s) responsible for MSC-NP-mediated trophic effects to better define possible mechanisms of repair.

A substantial risk associated with any stem cell therapy is the potential for unrestricted or ectopic growth, which can arise because of the heterogeneity and/or genomic instability of stem cell populations. MSCs, which have multipotential differentiation capabilities, are inherently heterogeneous [23, 43] and have been associated with ectopic connective tissue differentiation upon CNS transplantation in EAE mice [18]. Although recent clinical trials in MS patients suggested that intrathecal administration of autologous MSCs was safe [44–46], the findings in EAE nevertheless highlight the risk associated with nonhomologous application of heterogeneous cell populations [47]. Our analysis with neural-committed human MSC-NPs showed that only a small fraction of MSC-NPs retain adipogenic or osteogenic differentiation potential compared with MSCs, indicating that reduced mesodermal differentiation capacity and increased homogeneity of MSC-NPs may decrease the risk of ectopic differentiation upon CNS transplantation. Indeed, unlike with MSCs, there were no abnormal cellular masses found after intrathecal injection of syngeneic MSC-NPs into EAE mice [15].

Stem cell transplantation strategies carry the additional risk of uncontrolled and possibly tumorigenic growth arising from spontaneous chromosomal aberrations that occur during expansion in culture. The degree of genomic stability of ex vivo expanded adult MSCs is still a matter of some debate, with some studies showing that human MSCs maintain a normal karyotype over a significant number of passages [48–50], whereas more recent studies have detected a consistent 4% rate of chromosomal aberrations occurring even at early passages of MSCs [51, 52]. Karyotyping of bone marrow MSC lines in our study detected chromosomal aberrations in 1 of 11 cell lines. The chromosomal
rearrangement was evident at an early passage, but it was not clear whether this conferred any growth advantage to the MSCs. It is likely that the chromosomal abnormality occurred as a consequence of ex vivo expansion, because repeat bone marrow aspiration and isolation of MSCs revealed a normal karyotype. Our findings underscore the necessity for continuous monitoring of the genetic stability of MSC-based products, as well as a better understanding of how chromosomal aberrations might compromise safety of these products as they move into clinical use.

If a cell therapy-based approach is to be translated into clinical trials, a number of preclinical studies are necessary to identify the source of cells (adult or embryonic), the optimal route of administration, dosing regimen, and long-term safety, tolerability, and efficacy [15]. Initial studies in MS have used MSCs given intravenously or intrathecally and appear well tolerated [44–46]. MSC-NPs represent a neurally committed source of MSCs that have shown efficacy in EAE [15], and the current study shows that they are qualitatively suitable for consideration of use in human trials. It is likely that the use of these cells alone may not result in dramatic clinical improvement in the initial clinical studies. One envisions that eventually autologous stem cells would be used in combination with growth-related hormones and proteins or differentiation factors, such as anti-LINGO (LRR and Ig domain-containing, Nogo receptor-interacting protein), to establish a therapeutically created trophic environment.

**CONCLUSION**

This study investigated the feasibility of using human adult MSC-NPs for therapeutic application in neurological diseases. Specifically, we have shown that MSC-NPs are a subpopulation of MSCs that exhibit neural progenitor characteristics based on morphological and gene expression analysis. Furthermore, we showed that MSC-NPs are similar to MSCs with regard to their immunosuppressive and trophic properties but have the advantage of minimizing the risk of ectopic mesodermal differentiation. We also showed that MSCs and MSC-NPs derived from patients with MS were comparable to cells derived from control donors in terms of phenotype and therapeutic potential in vitro. These data, together with our previous work in an experimental model of MS, provide preclinical support for the use of autologous bone marrow-derived MSC-NPs as a source for cell-based therapy in MS.

**ACKNOWLEDGMENTS**

This work was supported by the Emerald Foundation and the Damal Foundation.

**AUTHOR CONTRIBUTIONS**

V.K.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; R.F. and T.V.: collection and/or assembly of data, data analysis and interpretation; S.A.S.: conception and design, financial support, provision of study material or patients, data analysis and interpretation, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTERESTS**

The authors indicate no potential conflicts of interest.

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