

INTRODUCTION

The severe neurological disability in multiple sclerosis (MS) is a result of degeneration of neurons and myelin-producing oligodendrocytes following autoimmune attack. There is an urgent need for therapies that can reverse the disability associated with MS.

Mesenchymal stromal cells (MSCs) are multipotential adult stem cells that exhibit neural differentiation potential, have an immunoregulatory function, and can promote the genesis of neurons and oligodendrocytes from neural stem cells. MSCs are extracted from bone marrow aspirates and are a particularly attractive source of cells for repair of CNS damage. This study examines whether bone marrow-derived mesenchymal stem cells (MSCs) are a viable source of neural progenitors for autologous stem cell therapy in multiple sclerosis (MS). Our experimental goal is to map out MSC-based repair strategies in a non-human primate experimental autoimmune encephalomyelitis (EAE) model of MS, and to extend these studies to human subjects.

METHODS

Human bone marrow aspirates were obtained from patient volunteers at the International Multiple Sclerosis Management Practice (IMSP) through an IRB-approved protocol. Control (non-MS) bone marrow samples were purchased from Lonza. Marmoset bone marrow aspirates were obtained from the proximal end of the surgically-exposed femur. MSCs were isolated from the mononuclear cell fraction of bone marrow by gradient centrifugation and enriched based on their plastic adherent properties. Expansion was carried out in MSCBM medium (Lonza) containing 10% autologous serum, or 10% FBS, and passaged at a density of 2000 cells/cm². For neural precursor differentiation, MSCs were incubated in serum-free neuronal progenitor maintenance media (NPM, Lonza) containing bFGF (20 ng/ml) and EGF (20 ng/ml) for 10-15 days. For differentiation assays, cells were incubated in adipogenic medium or osteogenic medium (both from Lonza) for 3 weeks. For neural differentiation, neurospheres were plated on matrigel-coated slides in the presence of bFGF (100ng/ml) for 3 weeks. Protein expression was detected by standard immunofluorescence. RNA was purified by RNeasy (Qiagen), cDNA synthesized by Superscript III (Invitrogen) and real-time quantitative PCR carried out using gene-specific primers and detected by SYBR Green (Roche).

RESULTS

Bone marrow-derived MSCs were isolated from human control and MS patients, and from marmosets. All MSCs showed characteristic spindle shaped morphology (Fig 1A and B) and were confirmed CD105+/CD90+/CD73+/CD34-/CD45-/CD14-/CD19-/HLA-DR- by FACS analysis (not shown). Both human and marmoset MSCs differentiated into adipocytes and osteocytes (Fig 1C-E). MSCs from MS patients were initially expanded in 10% autologous serum (AS). No difference in morphology, differentiation capability, or surface marker expression was noted. Human MSCs expanded in AS for up to 6 passages yielding an average of 20 million cells. Karyotype analysis after 6 passages showed no chromosome instability (Fig 1F).

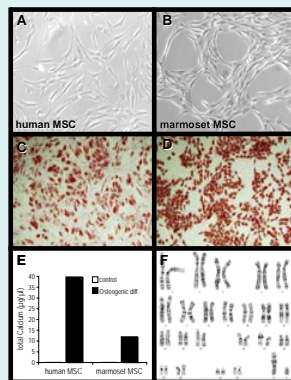


Figure 1. Spindle-shaped morphology of human (A) and marmoset (B) MSCs isolated from bone marrow as viewed by 10X light microscopy. (C, D) Differentiation of human (C) and marmoset (D) MSCs into adipocytes and stained with Oil Red O. (E) Osteogenic differentiation of MSCs shown by increased Calcium content. (F) Karyotype analysis of human MSCs from a multiple sclerosis patient showing normal karyotype after 6 passages in autologous serum.

Long-term culture of MSCs (Figure 2) in 10% FBS demonstrated that after approximately 15 population doublings, most human MSCs (both control and MS) displayed a flattened, enlarged morphology and became senescent. A subset of MSCs from both control and MS patients could be expanded greater than 200 days in culture (30-50 population doublings). Marmoset MSCs were expanded up to 250 days in culture, with over 100 population doublings. The difference in expansion capability of human compared to marmoset MSCs is probably due to the much longer telomeres found on marmoset chromosomes (not shown).

Investigation of the neural differentiation potential of MSCs showed that growth of MSCs in serum-free bFGF and EGF-containing media resulted in a striking morphologic transition from adherent cells to free-floating aggregates (Figure 3A and B), which are morphologically identical to neural precursors derived from the brain. These MSC-derived neural precursors (MSC-NP) showed increased levels of Nestin protein expression by immunofluorescence (not shown), increased levels of Nestin mRNA and decreased levels of Vimentin mRNA (Figure 3C). Nestin is a specific marker for neural stem cells, whereas Vimentin is a mesenchymal antigen, suggesting transition from MSC to a neural progenitor phenotype. MSC-derived NPs maintain the immunomodulatory properties similar to MSCs (Figure 3D).

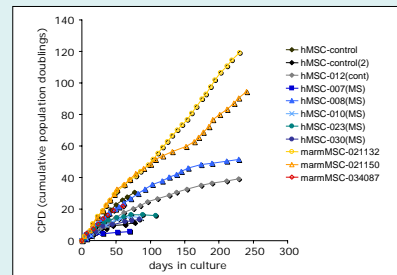


Figure 2. Long-term growth of MSCs in culture. MSCs from control or MS patients, or from marmosets were expanded in 10% FBS. For each passage, cells were re-plated at a density 2000 cells/cm².

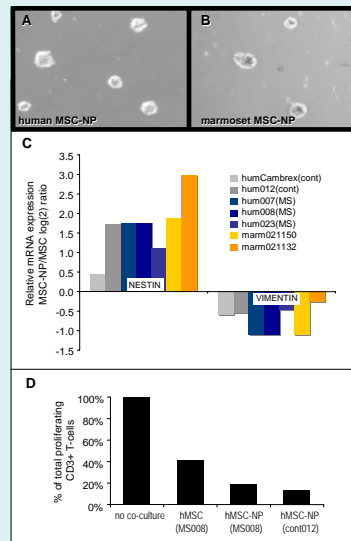


Figure 3. (A, B) Spherical morphology of human (A) and marmoset (B) MSC-derived neural precursors (MSC-NP) after culture for 15 days in EGF and bFGF. (C) Expression of Nestin increases and Vimentin decreases in MSC-NPs from human (control and MS) and marmosets. mRNA levels determined by quantitative PCR. Each value is normalized to RPLP and expressed as the ratio log(MSC-NP/MSC) log(2). (D) CFSE-labeled allogeneic T cells from peripheral blood were stimulated with PHA for 4 days. T cells were cultured alone, or co-cultured with human MSCs (from MS patient #008), MSC-NP (#008), or MSC-NP/control patient #012). Proliferation was measured by FACS analysis based on decreased CFSE fluorescence.

MSC-NPs were further differentiated in the presence of high dose bFGF. Differentiated cells exhibit a neuronal-like morphology with condensed cell body and multiple processes (Figure 4). MSC-NPs stained positive for neuronal (β -tubulin) and oligodendroglial (O4, O1, and GalC) lineage markers (Figure 4). These data suggest that MSC-NPs are able to differentiate *in vitro* into immature neuronal and oligodendroglial cell types. Further experiments are ongoing in our laboratory in order to determine whether these cells can further differentiate into mature, functional neurons or oligodendrocytes.

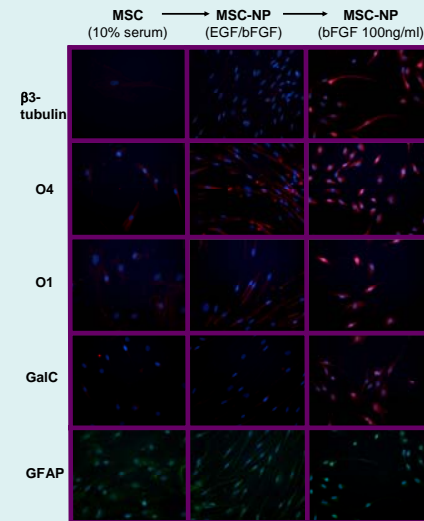


Figure 4. Increased neuronal and oligodendroglial lineage marker expression in differentiated MSC-NPs. Human MSC-NPs incubated for 3 weeks in either control NPM(EGF+bFGF), or neuronal differentiation medium containing 100 ng/ml bFGF. MSCs were cultured in 10% FBS. Protein expression was detected by immunofluorescence.

CONCLUSIONS

- Marmoset MSCs can be efficiently isolated from bone marrow, expanded *ex vivo*, and differentiated toward a neural precursor lineage. Ongoing studies are designed to determine whether autologous injection of these cells can safely induce repair in marmoset EAE.
- Human MSCs derived from multiple sclerosis patients exhibit identical morphology, growth, and differentiation characteristics as non-MS controls, demonstrating that multiple sclerosis has no effect on MSCs. These findings suggest that autologous MSCs are a potential source for cell-based therapy in MS.
- MSCs can be differentiated into neural precursors *in vitro* and are capable of further development into neuronal and oligodendrocyte lineage cell types, suggesting that MSCs may have the potential for neural repair.
- Future studies will determine the feasibility of using autologous MSC-NP to promote neural repair in human subjects.