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NEURAL TRANSDIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS ON HYDROPHOBIC POLYMER-MODIFIED SURFACE AND THERAPEUTIC EFFECTS IN AN ANIMAL MODEL OF ISCHEMIC STROKE

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Abstract—Human bone marrow-derived mesenchymal stem cells (MSCs) have multi-lineage differentiation potential and can become cells of mesodermal and neural lineages. These stem cells thus hold considerable clinical promise for the treatment of neurodegenerative diseases. For successful regeneration of damaged neural tissues, directed differentiation of neural or neuronal precursor cells from MSCs and integration of transplanted cells are pivotal factors. We induced MSCs into neurogenesis using a modified protocol. The therapeutic potency of the resulting neural progenitor cells in a rat model of ischemic stroke was analyzed. Using a highly hydrophobic diphenylamino-*s*-triazine-bridged *p*-phenylene (DTOPV)-coated surface and adopting a procedure for propagation of neural stem cells, we efficiently converted MSCs into neurosphere-like cellular aggregates (NS-MSCs). The spherical cells were subsequently induced to differentiate into neural cells expressing neuroectodermal markers. To determine whether these cells had neuronal fates and induced neuro-protective effects *in vivo*, NS-MSCs were intra-cerebrally administered to rats 48 h after permanent middle cerebral artery occlusion (pMCAo). The results showed a remarkable attenuation of ischemic damage with significant functional recovery, although the cells were not fully incorporated into the damaged tissues on post-operative day 26. Improvement in the NS-MSC-transplanted rats was faster than in the MSC group and suppression of inflammation was likely the key factor. Thus, our culture system using the hydrophobic surface of a biocompatible DTOPV coating efficiently supported neural cell differentiation from MSCs. Neural-primed MSCs exhibited stronger therapeutic effects than MSCs in rat brains with pMCAo.

Key words: bone marrow, DTOPV, differentiation, MSC, neural stem cells, neurosphere.

INTRODUCTION

Stroke is characterized as the rapid loss of brain function caused by a lack of blood supply to the brain and is a major cause of death or permanent disability. Ischemic stroke, the most common type of cerebrovascular injury, accounts for over 80% of all strokes. Ischemia in the brain causes local and/or global neuronal death upon oxygen deprivation, leading to permanent loss or impairment of body function (Donnan et al., 2008). Although thrombolytic approaches are available for acute phase stroke, this immediate treatment is often impossible in clinical settings. Despite enormous pharmacological and medical advances, effective treatments for stroke after the acute phase are still needed. Cell therapy with various types of progenitor/stem cells is emerging as a potential strategy for the treatment of stroke and other neurological disorders. These cells include neural cells from the fetal brain, and neural stem/progenitor cells derived from embryonic stem cells and adult stem cells. The clinical use of fetal tissue or cells from embryonic stem cells is limited due to ethical and technical problems as well as histocompatibility issues. Alternatively, adult stem cells from autologous sources are becoming leading candidates for regenerative medicine.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells with the capacity to differentiate into several cell types of tissues such as osteoblasts, adipocytes, chondrocytes, and neural cells (Pittenger et al., 1999; Chopp et al., 2000; Cho et al., 2005). The multipotency of MSCs has led to studies on their efficacy as a

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Abbreviations: 7-AAD, 7-aminoactinomycin D; BSA, bovine serum albumin; COMP, cartilage oligomeric matrix protein; CFSE, carboxyfluorescein succinimidyl ester; DAPI, 4′6-diamidino-2-phenylindole; DCX, doublecortin; DTOPV, diphenylamino-*s*-triazine-bridged *p*-phenylene vinylene; EDTA, ethylenediamminetetraacetate; FBS, fetal bovine serum; hESC, human embryonic stem cell; mNSS, modified neurological severity score; MSC, mesenchymal stem cells; NSC, neural stem cells; NS-MSC, neurosphere-like aggregates from MSCs; PBS, phosphate-buffered saline; PFA, paraformaldehyde; pMCAo, permanent middle cerebral artery occlusion; P/S, penicillin/streptomycin; PSA-NCAM, polysialic acid-neural cell adhesion molecule.

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therapeutic tool for various human diseases including neurodegenerative disorders (Bang et al., 2005; Mazzini et al., 2012). A number of studies have reported that MSCs can treat neurodegenerative diseases such as stroke or Parkinson's disease and have therapeutic potential for stroke (Li et al., 2001; Kurozumi et al., 2005). The major advantages of MSCs are the ease of large-scale, clinical-grade *in vitro* culture, they are of an autologous source with little or no concern of tissue rejection, and their safety regarding tumor formation. In addition, MSCs express various neurotrophic factors that can promote neurogenesis in the injured brain (García et al., 2004; Chen et al., 2005). While MSCs improve functional recovery without adverse effects in animal models and in human trials (Locatelli et al., 2009; Lee et al., 2010), they require further optimization. One such pivotal factor in MSC-based cell therapy for neurodegenerative disease is the neural differentiation from MSCs.

To date, various studies have reported that MSCs cultured under proper conditions can change phenotypes and express markers of neural cells through use of cytokines/growth factors, chemicals, and combinations of both (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Deng et al., 2001; Hermann et al., 2004; Cho et al., 2005; Tondreau et al., 2008). However, chemically induced neural differentiation has been questioned regarding the exposure of MSCs to chemicals, which lead to culture artifacts due to cytoskeletal disorganization, cell shrinkage, and particularly cytotoxicity (Lu et al., 2004; Phinney and Prockop, 2007). As the action of these chemical agents is quick and reversible, differentiated neural-like cells were unstable and non-viable *in vitro* as well as *in vivo.* In contrast, cytokine/growth factor-based approaches are more reliable and have yielded promising results (Cho et al., 2005; Khoo et al., 2008). While studies showed that MSCs can be differentiated into neuron-like cells *in vivo* and improve neurological function after cerebral ischemia (Chopp and Li, 2002), the survival rate and efficacy of simple transplantation of MSCs in ischemic tissue is very low (Phinney and Prockop, 2007). Therefore, more reliable and stable induction methods that do not affect cellular integrity are required for MSC neurogenesis. In the present study, we explored whether the physical property of diphenylamino-s-triazine-bridged *p*-phenylene vinylene (DTOPV) could efficiently induce neural/neuronal differentiation of human bone marrow-derived MSCs. While studies have shown that MSCs on the conventional tissue culture plate rapidly adhere to the hydrophilic surface and are refractory to neural induction (Mitchell et al., 2005), only 8% of human MSCs were converted into neurosphere-like aggregates, even with a low-attachment surface (Fu et al., 2008). Since neural stem cells (NSCs) possess a self-renewal capacity in a serum-free condition as floating aggregates which is a key step for neural induction (Hermann et al., 2004; Suzuki et al., 2004), we hypothesized that culturing cells with serum-free media on a non-adherent and extremely hydrophobic surface of unmodified DTOPV polymer-coated plate might facilitate differentiation of MSCs to NSC stages by forming neurosphere-like aggregates with high efficiency. With this approach, a large quantity of viable neurosphere-like aggregates from MSCs (NS-MSCs for human marrow-derived neurosphere-like structure forming cells) could be generated and further primed to neural cells with neurotrophic media. Finally, we showed that NS-MSCs significantly enhanced behavioral recovery and reduced the ischemic damage of permanent middle cerebral artery occlusion (pMCAo) in rats compared to MSC-transplanted rats possibly via the sustained action of transplanted NS-MSCs *in vivo*.

EXPERIMENTAL PROCEDURES

Reagents

DMEM (low glucose), fetal bovine serum (FBS), penicillin/streptomycin (P/S), trypsin/EDTA, phosphate-buffered saline (PBS), N2 supplement, B27 supplement, Trizol and Trypan Blue were purchased from Invitrogen (Carlsbad, CA, USA). DMEM/F12 was purchased from Lonza (Walkersville, MD, USA), and recombinant human bFGF, EGF, NT-3, GDNF, and BDNF were obtained from Peprotech (London, UK). Cyclosporin A was purchased from Calbiochem (San Diego, CA, USA). Fibronectin from human plasma, bovine serum albumin (BSA), triton X-100, silver nitrate, Oil Red O, Safranin-O, 7-aminoactinomycin D (7-AAD) and 4′6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tuj-1 antibody was purchased from Covance, and anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA, USA). Anti-Nestin, NeuN, polysialic acid-neural cell adhesion molecule (PSA-NCAM) and GFAP were purchased from Chemicon international (Temecula, CA, USA). Anti-ED1 (CD68) and doublecortin (DCX) were from Abcam (Cambridge, MA, USA). Anti-Ki-67 was from Leica Microsystems (Deerfield, IL, USA) and anti-rabbit IgG was from Vector Laboratories. Anti-mouse and anti-rabbit Cy3 were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Molecular Probes, Invitrogen (Eugene, OR, USA). Paraformaldehyde (PFA) solution (4%) was purchased from Biosesang (Seongnam, Korea).

Cell preparation

Human bone marrow from healthy adult volunteers was prepared by aspiration from the posterior iliac crest after informed consent was obtained (approval No. 4-2008-0643). Bone marrow mononuclear cells were isolated using density gradient centrifugation (GE Healthcare, Uppsala, Sweden) and were plated at a density of 1×10^6 cells/cm^2 in DMEM supplemented with 10% FBS, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, non-adherent cells were washed and remaining adherent cells were then cultured in fresh medium until they reached 70–80% confluence. The cells were then harvested by trypsinization and subcultured at a density of 5000 cells/cm^2. The medium was changed every third day and cells were subcultured using 0.05% trypsin/EDTA when they reached 90% confluence. The adherent MSCs at passage 3–5 were used for this study.

Fabrication and characteristics of DTOPV

DTOPV film was made using the Wittig polycondensation method as reported previously (You et al., 2009). DTOPV film was prepared by spin coating with a chloroform solution of DTOPV