Experimental Neurology 261 (2014) 53–64

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury

Vanessa Donega [a], Cora H. Nijboer [a], Geralda van Tilborg [b], Rick M. Dijkhuizen [b], Annemieke Kavelaars [c], Cobi J. Heijnen [a,c,*]

[a] Lab. of Neuroimmunology and Developmental Origins of Disease, University Medical Center Utrecht, Utrecht, The Netherlands
[b] Biomedical MR Imaging and Spectroscopy Group, Image Sciences Institute, University Medical Center Utrecht, The Netherlands
[c] Lab. of Neuroimmunology, Department of Symptom Research, The University of Texas, MD Anderson Cancer Center, Houston, TX, USA

ARTICLE INFO

Article history:
Received 18 February 2014
Revised 30 May 2014
Accepted 9 June 2014
Available online 16 June 2014

Keywords:
Neonatal hypoxia–ischemia
Nasal MSC therapy
Microglia polarization
Neurogenesis
Neuroregeneration
Scar formation

ABSTRACT

Previous work from our group has shown that intranasal MSC-treatment decreases lesion volume and improves motor and cognitive behavior after hypoxic–ischemic (HI) brain damage in neonatal mice. Our aim was to determine the kinetics of MSC migration after intranasal administration, and the early effects of MSCs on neurogenic processes and gliosis at the lesion site.

HI brain injury was induced in 9-day-old mice and MSCs were administered intranasally at 10 days post-HI. The kinetics of MSC migration were investigated by immunofluorescence and MRI analysis. BDNF and NGF gene expression was determined by qPCR analysis following MSC co-culture with HI brain extract. Nestin, Doublecortin, NeuN, GFAP, Iba-1 and M1/M2 phenotypic expression was assessed over time.

MRI and immunohistochemistry analyses showed that MSCs reach the lesion site already within 2 h after intranasal administration. At 12 h after administration the number of MSCs at the lesion site peaks and decreases significantly at 72 h. The number of DCX^+ cells increased 1 to 3 days after MSC administration in the SVZ. At the lesion, $GFAP^+$/$nestin^+$ and DCX^+ expression increased 3 to 5 days after MSC-treatment. The number of $NeuN^+$ cells increased within 5 days, leading to a dramatic regeneration of the somatosensory cortex and hippocampus at 18 days after intranasal MSC administration. Interestingly, MSCs expressed significantly more BDNF gene when exposed to HI brain extract *in vitro*. Furthermore, MSC-treatment resulted in the resolution of the glial scar surrounding the lesion, represented by a decrease in reactive astrocytes and microglia and polarization of microglia towards the M2 phenotype.

In view of the current lack of therapeutic strategies, we propose that intranasal MSC administration is a powerful therapeutic option through its functional repair of the lesion represented by regeneration of the cortical and hippocampal structure and decrease of gliosis.

Introduction

Encephalopathy caused by neonatal hypoxic-ischemic (HI) brain injury results in cerebral tissue loss leading to long-term neurological deficits *e.g.* mental retardation and motor impairment (De Haan et al., 2006; Ferriero, 2004; Graham et al., 2008; van Handel et al., 2007; Volpe, 2001).

The capacity of stem cells to treat neonatal encephalopathy is gaining support from an increasing number of studies (Bacigaluppi et al., 2009; Daadi et al., 2010; Donega et al., 2013a; Lee et al., 2010; Pimentel-Coelho et al., 2010; Titomanlio et al., 2011; Yasahura et al., 2008; van Velthoven et al., 2010, 2011, 2013). These studies describe the therapeutic potential of intracranially and intravenously delivered neural stem cells (NSCs) or mesenchymal stem cells (MSCs) in rodent models of neonatal HI or neonatal stroke. We have shown recently that both intracranial and intranasal MSC-treatment at 10 d after HI in neonatal mice significantly decreases cerebral lesion volume and improves long-term motor and cognitive behavior (Donega et al., 2013a; van Velthoven et al., 2010).

In view of the therapeutic potential of non-invasive intranasal MSC administration, we investigated the mechanism underlying MSC-mediated repair. Firstly, we studied the kinetics of MSC migration to the lesion site after intranasal administration. To visualize the arrival of MSCs in the brain, we used fluorescence microscopy and Magnetic Resonance Imaging (MRI). We determined the short- and long-term effects of MSCs on regeneration of the lesion by systematic quantification and characterization of precursor cells (type B cells; uncommitted

* Corresponding author at: Lab of Neuroimmunology, Department of Symptom Research, The University of Texas, MD Anderson Cancer Center, 1515 Holecombe Blvd, Unit 384, FCT11.5060, Houston, TX 77030-4009, USA.
E-mail address: CJHeijnen@mdanderson.org (C.J. Heijnen).

http://dx.doi.org/10.1016/j.expneurol.2014.06.009

precursors), neural progenitor cells (type A cells; neuronally-committed), neurons, microglia and astrocytes.

Material and methods

Ethics statement

Experiments were performed according to the international guidelines from the EU Directive 2010/63/EU for animals experiments and approved by the Experimental Animal Committee Utrecht (University Utrecht, Utrecht, Netherlands).

Animals

Unilateral HI brain damage was induced in 9 day old C57BL/6 mice (Harlan Laboratories, The Netherlands) by permanent occlusion of the right common carotid artery under isoflurane anesthesia followed by hypoxia (45 min at 10% oxygen). Sham-controls underwent anesthesia and incision only.

MSCs were purchased from Invitrogen (GIBCO mouse C57BL/6 MSCs, Life Technologies, UK) and cultured according to the manufacturer's instructions. Characterization of cell specific antigens has been described previously by us (van Velthoven et al., 2011). Before administering 1×10^6 MSCs intranasally, each nostril was treated with 3 μL of hyaluronidase (100 U, Sigma-Aldrich, St. Louis, MO) in PBS to increase the permeability of the nasal mucosa. Thirty minutes later, pups received 3 μL of MSCs or PBS (vehicle) twice in each nostril.

Histology

Coronal paraffin sections (8 μm) of paraformaldehyde (PFA)-fixed brains were incubated with mouse-anti-myelin basic protein (MBP) (Sternberger Monoclonals, Lutherville, MD,) or mouse-anti-microtubule-associated protein 2 (MAP2) (Sigma-Aldrich) followed by biotinylated horse-anti-mouse antibody (Vector Laboratories, Burliname, CA). Binding was visualized with Vectastain ABC kit (Vector Laboratories) and diaminobenzamidine.

Immunohistochemistry

MSCs were labeled with PKH-26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich). Coronal frozen sections (8 μm) were incubated overnight at 4 °C with primary antibodies; goat anti-DCX (1:300) (Santa Cruz Biotechnology, TX, USA), rabbit anti-Iba1 (1:500) (Wako Chemicals, Osaka, Japan), mouse anti-GFAP (1:100) (Acris antibodies, Herford, Germany), mouse anti-NeuN (1:200) (Chemicon, Temecula, CA), mouse anti-nestin (1:200) (BD Biosciences, Breda, The Netherlands), rat anti-CD16/CD32 (1:300) (BD Pharmingen, Breda, The Netherlands), goat anti-CD206 (1:300) (R&D Systems, Abingdon, UK). Primary antibody binding was detected by incubating with corresponding secondary antibodies for 1 h at room temperature (Supplementary Table 1). Nuclei were counterstained with DAPI (Invitrogen, Paisley, UK) and mounted with FluoroSave reagent (Calbiochem, Nottingham, UK). Fluorescent images were captured using an EMCCD camera (Leica Microsystems, Benelux) and Softworx software (Applied Precision, Washington, USA) or an AxioCam MRm (Carl Zeiss, Sliedrecht, The Netherlands) on an Axio Observer Microscope with Axiovision Rel 4.6 software (Carl Zeiss).

MSC labeling for MRI

Culture flasks were coated with Poly-L-Lysine (0.02 mg/mL) before seeding MSCs. 48 h later, MSCs were incubated with 0.01 mg Fe/mL fluorescent micron-sized superpara-magnetic iron-oxide particles (MPIO; 0.86 μm) (Bangs Laboratories Inc., IN, USA) diluted in GlutaMAX DMEM medium (Life Technologies). After 4 h, excessive MPIO particles were removed by washing 4 times with PBS. About 70% of the cells were labeled with MPIO particles. Images were taken on an Axio-Observer microscope (Carl Zeiss Microscopy, Jena, Germany) with Axiovision rel. 4.6 software (Carl Zeiss Microscopy).

MRI

MRI was performed on a 9.4 T horizontal bore preclinical MRI system (Varian Inc., Palo Alto, CA). T_2^*-weighted gradient echo images of cells in agarose were acquired with TR/TE = 1000/15 ms and 100 μm × 100 μm × 200 μm spatial resolution. T_2^*-weighted gradient echo images of *ex-vivo* mouse brain were acquired with TR/TE = 40/15 ms and a voxel size of 75 μm in all directions.

To verify the detectability of MPIO-labeled MSCs with MRI, cells were homogeneously distributed in 0.4% agarose in PBS at concentrations between 0 and 1000 cells/μL. T_2^*-weighted gradient echo images were acquired with a Millipede™ coil (Varian Inc.), using the following parameters: TR = 1 s, TE = 15 ms, flip angle = 90°, 4 averages, field-of-view 25.6 mm × 25.6 mm, matrix size 256 × 256 and slice thickness 0.2 mm. For the detection of MPIO-labeled cells in *ex-vivo* mouse brain, mice were perfused transcardially with 4% PFA at 2 h after MSC-treatment. T_2^*-weighted images were acquired with a 3D GE sequence, with TR = 40 ms, TE = 15 ms, flip angle = 15°, 32 averages, field-of-view = 22 mm × 12 mm × 10 mm and a voxel size of 75 μm in all directions.

MSCs co-culture with brain extracts

10 days after HI- or sham-operation, mice were euthanized by pentobarbital overdose and decapitated, and their brains were removed. The ipsilateral hemisphere was dissected on ice at −2.0–2 mm from bregma and was subsequently pulverized on liquid nitrogen. Dissected brains were dissolved in KO-DMEM medium (Life Technologies) at a final concentration of 150 mg/mL and centrifuged for 10 min at 3000 *g* at 4 °C. Supernatants were collected as 'brain extract' and protein concentration was measured with the protein assay (Bio-Rad, Hercules, CA). MSCs were cultured at a concentration of 40,000 cells per well in a 24 well-plate for 24 h before replacing the medium with knock-out medium containing 1 mg/mL brain extract. RNA was isolated from the MSCs 72 h after culture with brain extracts.

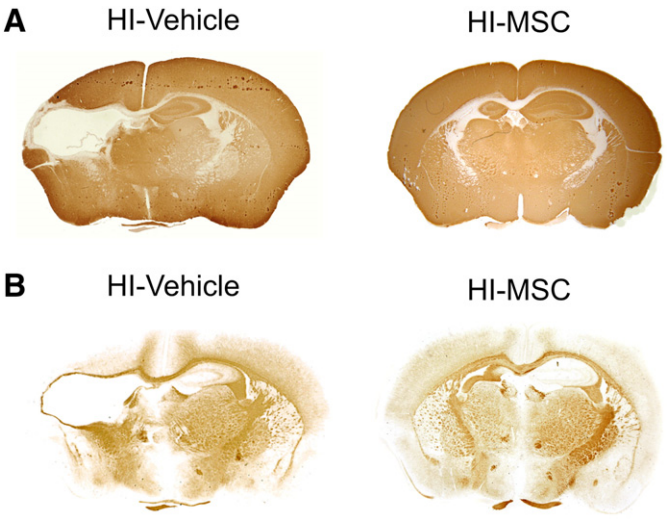

Fig. 1. Intranasal MSC treatment decreases HI lesion size. Representative images of (A) MAP2 and (B) MBP staining from HI-Vehicle and HI-MSC animals at 18 days after MSC administration (*i.e.* 28 days after HI).