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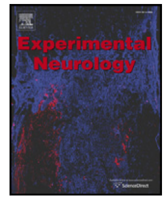

Regular Article

Diffuse and persistent blood–spinal cord barrier disruption after contusive spinal cord injury rapidly recovers following intravenous infusion of bone marrow mesenchymal stem cells

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ABSTRACT

Intravenous infusion of mesenchymal stem cells (MSCs) has been shown to reduce the severity of experimental spinal cord injury (SCI), but mechanisms are not fully understood. One important consequence of SCI is damage to the microvasculature and disruption of the blood spinal cord barrier (BSCB). In the present study we induced a contusive SCI at T9 in the rat and studied the effects of intravenous MSC infusion on BSCB permeability, microvascular architecture and locomotor recovery over a 10 week period. Intravenously delivered MSCs could not be identified in the spinal cord, but distributed primarily to the lungs where they survived for a couple of days. Spatial and temporal changes in BSCB integrity were assessed by intravenous infusions of Evans blue (EvB) with *in vivo* and *ex vivo* optical imaging and spectrophotometric quantitation of EvB leakage into the parenchyma. SCI resulted in prolonged BSCB leakage that was most severe at the impact site but disseminated extensively rostral and caudal to the lesion over 6 weeks. Contused spinal cords also showed an increase in vessel size, reduced vessel number, dissociation of pericytes from microvessels and decreases in von Willebrand factor (vWF) and endothelial barrier antigen (EBA) expression. In MSC-treated rats, BSCB leakage was reduced, vWF expression was increased and locomotor function improved beginning 1 week post-MSC infusion, *i.e.*, 2 weeks post-SCI. These results suggest that intravenously delivered MSCs have important effects on reducing BSCB leakage which could contribute to their therapeutic efficacy.

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Introduction

Traumatic spinal cord injury (SCI) results in changes in the microvasculature and disruption of the blood spinal cord barrier (BSCB). The initial breakdown of the BSCB is followed by secondary tissue damage caused by infiltration of immune cells and a variety of toxic molecules which contribute to neuronal loss, axon severing and demyelination commonly associated with SCI (Beck et al., 2010; Bartanusz et al., 2011). It is well established that the compromise of the BSCB after SCI persists for an extended period of time (Noble and Wrathall, 1989; Popovich et al., 1996). The reported time course of BSCB re-establishment after SCI, however, varies between studies likely due to the variety of tracers used to measure permeability. Noble and Wrathall (1989) used horseradish peroxidase to assess BSCB permeability and reported that the BSCB was re-established by 14 days after injury, while aminoisobutyric acid extravasation assays (Popovich et al, 1996) revealed a biphasic pattern of BSCB disruption, with secondary elevations of the tracer transfer in the spinal white matter 14 to 28 days post-SCI. Whetstone et al. (2003) reported acute changes in luciferase permeability in SCI mice as early as 35 min after injury with a secondary elevation in permeability 3–7 days after SCI. More recently, dynamic contrast-enhanced MRI in a rat model of SCI demonstrated a compromised BSCB as late as 56 days post-SCI (Cohen et al., 2009).

Given the contribution of BSCB disruption to ongoing pathology in SCI, repair of the BSCB has been considered a target site for SCI therapies (Horner et al., 1996). It is well-established that intravenous injections of mesenchymal stem cells (MSCs) can improve functional recovery in experimental models of SCI (Osaka et al., 2010; Quertainmont et al., 2012). In the present study we first characterized the spatial and temporal changes in the BSCB up to 10 weeks after SCI and then evaluated the effect of intravenous MSC delivery on vascular permeability. Contusive SCI resulted in a protracted increase in vascular leakage that extended

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centimeters rostral and caudal to the contusion site which, while reduced over time, persisted at the contusion site for at least 10 weeks. Intravenous MSC delivery resulted in reduced vascular leakage and improved locomotor function beginning 1 week after cell infusion (2 weeks post-SCI), indicating that intravenous MSCs may exert at least part of their therapeutic effect in SCI by accelerating restoration of BSCB integrity.

Methods

All experiments were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and the VA Connecticut Healthcare System Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

Contusive spinal cord injury (SCI)

Dorsal laminectomies (T9) were performed on adult male Sprague–Dawley rats (185–215 g) under isoflurane gas anesthesia, followed immediately by a delivery of 2 Newton impact (equal to 200 kilodynes) with a 2.5 mm tip at the T9 level using the Infinite Horizon (IH) impactor spinal cord injury device (Precision Systems & Instrumentation, Lexington KY). Appropriate post-operative care, including twice daily manual bladder expression for up to 14 days, and pain relief with buprenorphine (0.05 mg/kg/day SQ) for 48 h and ibuprofen (0.15 mg/ml = 5 mg/kg/day PO) for 72 h was provided for all animals.

Behavioral testing

Open field locomotor function was assessed using the Basso–Beattie–Bresnahan (B–B–B) locomotor rating scale (Basso et al., 1995) by a tester who was blinded with respect to treatment condition. Rats were scored 2 days prior to surgery, 3 days post contusion, 1 week post-contusion and at weekly intervals thereafter until the time of sacrifice 10 weeks post SCI.

Assessment of BSCB integrity with Evans blue (EvB)

Two hours prior to sacrifice with sodium pentobarbital anesthesia, a 4% solution of EvB (2.5 mg/kg) was infused into the femoral veins of uninjured control animals or contused rats at 1, 3, 7, 14, 28, 42, or 70 days post-contusion. After extensive perfusion with saline, spinal cords were removed and stripped of meninges and dye distribution was assessed by *ex vivo* imaging using an IVIS Lumina imaging system (Spectrum 200, Caliper Life Science Hopkins, MA) with the excitation and emission filters set at 620 and 680 respectively. For direct comparison of EvB fluorescence intensity between spinal cords, all images were taken at 0.25 s exposure and normalized to the intact condition by adjusting the Aggregate Color Scale to Min = 1.00e9 and Max = 3.00e9, such that the control cord appeared white and all colored areas indicated regions of increased dye extravasation above baseline.

To quantify the EvB dye content, three 1 cm segments of the spinal cord, one centered on the impact site (Lesion), and one each rostral (Rostral) and caudal (Caudal) to the lesion containing segment were removed, weighed, homogenized in 7.5% (w/v) trichloroacetic acid (TCA) and centrifuged for 10 min at 10,000 rpm 4 °C. Absorbance of the supernatant was measured at 620 nm using a microplate fluorescence reader (Synergy HT multilabel plate reader, Bio-Tek, Winosocki, Vermont) and EvB concentrations were calculated using standard curves obtained by serial dilution of EvB in 7.5% TCA with 4% calf serum, followed by subtraction of background values obtained for cords processed without EvB infusion. This method was validated by directly injecting known quantities of EvB into spinal cord tissue and comparing plate reader values to the known dye content. Values were expressed as μg of EvB/g spinal cord tissue. In intact control rats age matched for 1 day, 2 weeks and 10 weeks post-SCI no effect of age was observed on EvB extravasation in uninjured cords and data for all control cords was pooled.

Tissue processing and staining

For histological analysis, rats were deeply anesthetized with sodium pentobarbital and perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer and processed for standard frozen sectioning or with 2% paraformaldehyde and 2% glutaraldehyde and 1 μm semi thin plastic sections. Coronal (20 μm) sections taken near the centers of each block were stained with hematoxylin and eosin, (H&E) for gross histology or with two or more of the following primary antibodies diluted in 0.01% Triton X-100, 5% fishskin gelatin (0.1 M PBS) blocking buffer: mouse monoclonal RECA-1 (1/50; AbD Serotec MCA970R), rabbit monoclonal against PDGFR-β (1:200; Cell Signaling Technologies 3169S), chicken polyclonal anti-GFAP (1:1000; Encor CPCA-GFAP), rabbit polyclonal von Willebrand Factor (1:1000; ABCAM ab6994), or mouse monoclonal Endothelial Barrier Antigen (1:100; Covance SMI-71R) and visualized with secondary goat anti-mouse, -rabbit, or -chicken IgGs conjugated to Alexa Fluor 488, 546, 594, or 633 (Invitrogen, Eugene, OR; 1:1000) to identify microvessel localization, cell composition and gene expression. To evaluate trafficking of i.v. infused MSCs to contused cord, coronal sections of spinal cords of rats infused with i.v. MSCs were collected at the site of lesion, and 1 cm rostral and 1 cm caudal to the lesion and stained with chicken or rabbit anti-GFP antibodies (1:1000; Invitrogen A6455 or A10262). Semi thin plastic sections were stained with 0.5% methylene blue, 0.5% azure blue, and 0.5% borax. H&E stained frozen sections and methylene blue/azure blue stained plastic sections were cover slipped with Cytoseal 60 (Richard Allan Scientific) and examined and photographed with a Nikon Eclipse microscope. Immunostained sections were counterstained with DAPi mounting media (Vectashield, Vector Laboratories, Burlingame, CA) and photographed with a Nikon A1R multiphoton confocal microscope with NIS Elements software.

Analysis of microvessel density and sizes

For comparison of microvessel densities sizes, five 635 × 635 μm areas of interest were imaged at defined positions (dorsal, ventral, right lateral, left lateral, and center of the cord) in RECA-1 stained sections collected near the epicenter of the impact site or at the comparable position for intact cords. Vessel sizes and densities for control rats age matched for 1 and 10 week post SCI conditions showed no effect of age on vessel counts or size and data for both ages was pooled. Images were collected as 10 μm image stacks using a 20× objective lens on a Nikon A1R multiphoton confocal microscope and the diameters of individual vessels were then measured at their widest point using NIS Elements software. Vessels that branched within the image stack were counted and measured only once. Total vessel numbers and vessel sizes were analyzed for three animals each at 1, 2, 4, and 6 weeks post SCI, five animals at 10 weeks post SCI, and six intact controls (3 each age matched for 1 week and 10 weeks post SCI). Vessel count data for 1 and 2 weeks and 3 and 4 weeks post-SCI were pooled to increase n values for statistical analysis. To assess the relative size distribution of vessels, vessels were divided into 3 categories: small (0–7 μm), medium (7–14 μm), and large (≥14 μm) diameter. To assess whether the reductions in percentages of small diameter vessels in injured spinal cords could be caused entirely by small vessel loss, (as opposed to changes in vessel diameter), the predicted percentages of small diameter vessels which would be expected if the reductions in vessel

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