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Human Umbilical Cord Blood Cells Directly Suppress Ischemic Oligodendrocyte Cell Death

A.A. Hall^{1,†}, A.G. Guyer^{3,†}, C.C. Leonardo^{1,†}, C.T. Ajmo Jr¹, L.A. Collier¹, A.E. Willing^{1,2,†,*}, and K.R. Pennypacker^{1,†}

¹Department of Molecular Pharmacology and Physiology, School of Basic Biomedical Sciences, College of Medicine, University of South Florida, Tampa, FL 33612, USA

²Center for Excellence in Aging and Brain Repair, School of Basic Biomedical Sciences, College of Medicine, University of South Florida, Tampa, FL 33612, USA

³Department of Pathology and Cell Biology, School of Basic Biomedical Sciences, College of Medicine, University of South Florida, Tampa, FL 33612, USA

Abstract

Previous reports have shown that human umbilical cord blood cells (HUCBCs) administered intravenously 48 hrs following middle cerebral artery occlusion reduces infarct area and behavioral deficits of rodents. This cellular therapy is potentially neuroprotective and anti-inflammatory. This study investigates the effect of HUCBC treatment on white matter injury and oligodendrocyte survival in a rat model of ischemia. Intravenous infusion of 10⁶ HUCBCs 48 hrs post-stroke reduced the amount of white matter damage *in vivo* as seen by quantification of myelin basic protein staining in tissue sections. To determine whether HUCBC treatment was protective via direct effects on oligodendrocytes, cultured oligodendrocytes were studied in an *in vitro* model of oxygen glucose deprivation. Active caspase 3 immunohistochemistry and the LDH assay for cytotoxicity were used to determine that HUCBCs provide protection to oligodendrocytes *in vitro*. Based on these results, it is likely that HUCBC administration directly protects oligodendrocytes and white matter. This effect is likely to contribute to the increased behavioral recovery observed with HUCBC therapy.

Keywords

myelin; ischemia; stem cells; rat; stroke

Introduction

Stroke is a leading cause of death and disability in the United States (Wolf and Kannel 2007). In nearly all strokes, white matter is affected to some degree (Dewar et al. 2003), producing a significant injury since white matter accounts for nearly 50% of the total cortical volume in humans (Miller et al. 1980). Ischemic injury is a major cause of white matter dysfunction for reasons that are not yet fully understood. Experimental rodent models have aided in identifying many key substrates involved in exacerbating brain injury after ischemia. Potential mechanisms appear to involve disruption of axoglial junctions (Barres

*To whom correspondence should be addressed: Alison E. Willing, University of South Florida, Center for Excellence in Brain Repair, Box 78, 12901 Bruce B Downs Blvd., Tampa, Florida 33612, Phone: 813-974-7812, Fax: 813-974-3078, e-mail: awilling@health.usf.edu.

[†]Indicated authors contributed equally to this work.

and Raff 1999) which likely result from degradation of myelin proteins (Griffiths et al. 1998; Marcus et al. 2002), increased membrane permeability from reactive oxygen/nitrogen species (Lewen et al. 2000; Love 1999) and excitotoxicity associated with elevated extracellular glutamate (Back et al. 2007; David et al. 2003; Dingley et al. 2006; Follett et al. 2000).

Considerable neuronal and oligodendrocyte (OL) cell death occurs within a period of several days after stroke, and these effects are associated with increased neuroinflammation (Tanaka et al. 2001). To date, most studies evaluating treatments for ischemic injury have focused on neuronal survival and reduced inflammation as their primary end points. There is a paucity of studies, however, which evaluate treatments with respect to OL survival and white matter retention. Indeed, *in vitro* data shows that OLs are vulnerable to ischemia (Back et al. 2002; Lyons and Kettenmann 1998) and OL cell death is associated with impaired performance on motor tasks (Mabuchi et al. 2000; Pantoni et al. 1996). OL survival, in particular, is critical for the efficient saltatory conduction afforded by intact myelination of axonal tracts which is necessary for proper motor functioning.

Middle cerebral artery occlusion (MCAO) in rats has been used to mimic the progressive degenerative lesions that occur in humans following an embolic stroke. In this stroke paradigm, substantial neurodegenerative damage accompanied by robust immune cell recruitment is seen in the striatum, a white matter rich area in the rat brain. Intravenous infusions of HUCBCs up to 48 hours after MCAO have been shown to reduce infarct volume (Newcomb et al. 2006) and associated behavioral deficits (Chen et al. 2001; Vendrame et al. 2004; Willing et al. 2003). Additionally, HUCBCs improve the behavioral deficits that result from spinal cord injury (Saporta et al. 2003) and reduce the infarct volume associated with myocardial infarction (Henning et al. 2004; Henning et al. 2006).

Though originally thought to be a cell replacement therapy due to their stem cell properties, HUCBCs possess potent systemic anti-inflammatory properties which account for some of the observed behavioral improvements (Newman et al. 2006; Vendrame et al. 2005). It remains to be determined whether HUCBCs can directly afford protection to white matter structures and oligodendrocytes following ischemia. The purpose of the present study was to identify whether HUCBCs can ameliorate white matter damage and increase OL survival following ischemia.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 300 to 350g were used for MCAO surgeries. Timed pregnant female Sprague-Dawley rats were used for mixed glia preparations. All animals were housed in a climate controlled room with food and water provided *ad libitum*. Pregnant females were allowed to give birth and the pups were used at post-natal day 2. Animals were handled and cared for according to the guidelines set forth by the IACUC committee of the University of South Florida College of Medicine.

Laser Doppler Blood Flow Measurement

Prior to MCAO surgery, animals were anesthetized with 5% isoflurane/100% O₂ in an induction chamber. Rats were treated prophylactically with Ketophan (10 mg/kg IM), atropine (0.25 mg/kg SC) and Baytril (20 mg/kg IM) in accordance with IACUC guidelines. Ketophan injections were continued 3 days post-MCAO to minimize pain and discomfort. A constant flow of anesthesia was supplied with an interfaced scavenging system (3 to 4% isoflurane, flow rate 1 L/min) throughout the procedure. For Doppler insertion, the head was shaved and an incision was made lateral to the midline of the dorsal plates of the skull.

The skin was spread open and the tissue covering the skull bone was pushed aside with a cotton-tipped applicator. Using a microdrill, a small hole was drilled into the skull at 1 mm posterior and 4 mm lateral to bregma. A hollow stainless steel guide screw was screwed into the hole. A fiber optic filament (500 μ m) was then inserted through the screw guide and secured with Vetbond (3M, St. Paul, MN). Blood perfusion in the brain was monitored using the Moor Instruments (Devon, England) LTD laser Doppler with MoorLAB proprietary Windows-based software on a standard laptop. Once the MCAO surgery was complete, the screw guide was removed, bone wax was placed in the burr hole and the scalp incision was sutured. Rats that did not show $\geq 60\%$ reduction in blood perfusion during MCAO were excluded from the study.

Middle Cerebral Artery Occlusion and HUCBC Treatment

MCAO surgeries were performed on male Sprague-Dawley rats (300–350 gm) as described previously (Ajmo et al. 2006; Butler et al. 2002; Vendrame et al. 2004). Following implantation of the Doppler probe, the external carotid artery was exposed and isolated from the vagus nerve using blunt dissection. The artery was then ligated and transected near the bifurcation of the internal and external carotid arteries. The stump of the external carotid was then used as a guide to advance a monofilament through the internal carotid artery to the origin of the middle cerebral artery. The filament was then sutured into place and the incision closed. For sham surgeries, the Doppler probe was inserted and the external carotid was exposed, but no filament was inserted.

To determine whether HUCBCs were protective against stroke-induced white matter damage, rats were injected with either 10^6 HUCBCs or 500 μ L of normal saline (i.v.) 48 hours after MCAO surgery. The animals were then sacrificed 96 hours post-stroke, transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde. The brains were then removed and saturated with 4% paraformaldehyde followed by increasing concentrations of sucrose (20%, 30%). Brains were then sectioned on a cryostat at 30 μ m thickness and stored in Walter's Antifreeze at -20°C .

Mixed Glial Culture Preparation

Primary mixed glial cultures were prepared from Sprague-Dawley rats using a protocol previously described (Gottschall et al. 1995). Briefly, postnatal day 2 rat pups were sacrificed by decapitation. The brains were removed and the meninges dissected away. The exposed cortices were dissociated in a solution of 0.25% Trypsin/2.21mM EDTA, triturated to yield a single cell suspension and pelleted. The pellet was resuspended in DMEM (Mediatech, Manassas, Va) supplemented with 10% horse serum, 2.5% fetal bovine serum and 1% antibiotic/antimycotic (DMEM+). Viable cell yield was determined by Trypan Blue exclusion. Cells were seeded (1.5×10^7) into poly-L-Lysine - treated 75cm² tissue culture flasks. Fresh DMEM+ was added to flasks the following day and mixed glial cultures were incubated for 8–10 days at 37°C .

Purification of Oligodendrocyte Cultures

OLs were purified from 8–10 day old mixed glial preparations by mechanical dissociation as previously described (McCarthy and de Vellis 1980). Mixed glial cultures were shaken for 1 hour to separate OLs and microglia from the astrocyte monolayer. The media was removed and the cells were pelleted. The pellet was resuspended in DMEM+ and viable cells were counted using Trypan Blue exclusion. The microglia and OL-containing media was added into 10 cm plastic tissue culture dishes at a density of 10^7 cells/dish and incubated 15 min at 37°C to allow microglia to adhere to the plastic. Following incubation, the dishes were gently swirled and OL-containing media was collected. This differential adherence protocol was repeated twice to purify the cultures. The resulting cell suspension was pelleted,

resuspended in DMEM+ and plated on glass poly-L-lysine – treated coverslips at 10⁵ cells/coverslip. The following day, cell media was changed to Neurobasal Complete (Neurobasal supplemented with B-27 and L-glutamine (0.5 mM)) + 10 ng/ml PDGF AA (Barres et al. 1993; Yang et al. 2005). The oligodendrocytes remained in Neurobasal Complete + PDGF for 7 days to encourage proliferation. Following the proliferation period, PDGF was withdrawn for a period of 5 days to induce differentiation into the mature OL phenotype (Yang et al. 2005). OL cultures were used for experiments immediately following the 5 day PDGF AA withdrawal. Cell densities at the time of experiment averaged 5×10⁵ cells/coverslip.

Oxygen Glucose Deprivation

OL cultures plated on glass coverslips were randomly assigned to one of two experimental conditions: oxygen glucose deprivation (OGD) (DMEM minus glucose), or normoxia (DMEM minus glucose). Transwell inserts (0.4 µm; Millipore, Billerica, MA) were added to the 6-well plates containing the coverslips. Cryopreserved HUCBCs (Saneron CCEL Therapeutics, Inc., Tampa, FL) were rapidly thawed, washed and pelleted to remove the DMSO and resuspended in 10 ml of DMEM. The HUCBCs were seeded onto 6-well cell inserts at 10⁴ cells/insert and placed into the wells with the OL-containing coverslips immediately prior to OGD exposure. A negative control consisting of media alone was included for each experimental condition. In addition, wells containing 10⁴ HUCBCs alone were included as controls to quantify the amount of HUCBC death contributing to the LDH assay for each experimental condition.

Cells undergoing OGD were placed in an air-tight hypoxic chamber that was flushed with hypoxic gas (94% N₂, 5%CO₂, 1% O₂; Airgas, Tampa, FL) for 15 minutes and then sealed for the duration of the exposure. For normoxia, cells were maintained in a standard tissue culture incubator. Cultures were subjected to OGD or normoxia for 2 hrs at 37°C. Cells that received normoxia were maintained in their respective media. The media from each of the wells was then collected and stored at 4°C until the LDH assay was performed. The glass coverslips were fixed for 30 min in 4% paraformaldehyde for subsequent immunohistochemical assays.

Lactate Dehydrogenase (LDH) Assay

OL cell death in culture was determined using the LDH assay (Takara Bio, Inc., Madison, WI). Briefly, 100 µl of tissue culture media from each experimental group was added to a 96 well plate and 100 µl of LDH reagent was added to each well. Plates were incubated for 30 min at 25°C and absorbances were read on a microplate reader at 548 nm wavelength. The media from the HUCBC only wells served as a control of HUCBC death. The absorbance of HUCBC only media, as well as the absorbance of media only, was subtracted from the total absorbance of the OL wells to eliminate background LDH activity.

A standard curve was generated from experiments using known concentrations of OLs. In these experiments, the cells were lysed with 2% Triton X-100 and the amount of LDH release was quantified. The absorbances derived from the known concentrations were used to produce the standard curve. The reported OL cell numbers from the hypoxia experiments were extrapolated from this standard curve.

Immunohistochemistry

Brain tissue sections were washed three times in phosphate-buffered saline (PBS, pH 7.4) to remove Walter's antifreeze and then mounted onto slides and dried. Both the slides and OL-containing coverslips were washed in PBS and incubated for 1 hr at 25°C in permeabilization buffer containing 2% goat serum, 0.3% Triton X-100 and 0.3% 1M lysine

in PBS. Samples were then incubated overnight at 4°C in primary antibody solution (2% goat serum, 0.3% Triton X-100 in PBS). The following day, all samples were rinsed in PBS and incubated 1 hr at 25°C in secondary antibody solution (2% goat serum and 0.3% Triton X-100 in PBS). Double-label immunohistochemistry was achieved by co-incubation with anti-rat, anti-mouse or anti-rabbit primary antibodies, and subsequent co-incubation with secondary antibodies conjugated to distinct fluorophores for each respective species of primary antibody. OL-containing coverslips and slide-mounted tissue sections were washed in PBS and mounted onto slides or coverslipped using VectaShield (Vector Laboratories, Burlingame, Ca.) mounting media, respectively.

Rat anti-myelin basic protein (MBP) (Abcam, Cambridge, MA; 1:1000) was used as the primary antibody in the *in vivo* experiments. The primary antibodies used in the *in vitro* experiments consisted of the following: rat anti-myelin basic protein (MBP) (Abcam, Cambridge, MA; 1:1000); mouse anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Chemicon, Temecula, CA; 1:1000); mouse anti-O1 (Chemicon; 1:1000); rabbit anti-NG2 (Chemicon; 1:500); mouse anti-OX-42 (Serotec, Oxford, UK; 1:3000); and rabbit anti-active caspase 3 (Sigma, St. Louis, MO; 1:1000). Fluorescent detection was achieved using Alexa Fluor 488 (green) or 594 (red) secondary antibodies (Molecular Probes, Eugene, OR; 1:1000) corresponding to the respective species of primary antibodies.

Image Analyses

For *in vivo* image analyses, brain sections from a minimum of four animals per group were used. Three representative coronal brain sections encompassing the striatum (Bregma coordinates +1.7→−0.3) were taken from each animal and processed immunohistochemically. Images were acquired using a Zeiss Axioscope 2 microscope controlled by Openlab (Improvision Ltd, Lexington, MA) software. Photomicrographs were captured with a Zeiss Axicam Color camera. The Image-Pro Plus program (Media Cybernetics Inc., Bethesda, MD) was used to generate relative fluorescent intensities and the data was quantified and analyzed using GraphPad Prism 4.0.

For *in vitro* analyses, 9 separate fields per coverslip were photographed using the same system as in the *in vivo* analyses. Cell counts were performed using NIH ImageJ software. Cell count data were quantified and analyzed using GraphPad Prism 4.0.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4.0 for all experiments. For the *in vitro* studies, an “n” of 3 represents 3 separate experiments consisting of 3 replicate wells per group. One way ANOVA was used to determine whether there was a significant difference between treatments and a Tukey post hoc test was used to further analyze main effects. A “p” value of less than 0.05 was deemed to represent significant differences for the statistical analyses.

Results

Delayed Infusion of HUCBCs Reduces White Matter Damage after MCAO

MBP immunoreactivity has been used in previous studies to demonstrate white matter injury in rodents subjected to ischemia via carotid artery ligation (Jelinski et al. 1999). In this study, we used the rat MCAO model to induce ischemia and examined the corpus striatum for intact striosomes labeled with MBP. Intravenous infusions of HUCBCs were administered 48 hrs after MCAO surgery as described previously (Chen et al. 2001; Vendrame et al. 2004; Willing et al. 2003) and most effective dose at this time point is 10⁶ cells (Newcomb et al. 2006). Tissue sections were immunostained for MBP and the results

were compared to both MCAO only and sham surgery controls. Contralateral hemispheres for all groups served as internal controls to which the ipsilateral area of MBP staining was compared. In the MCAO treated animals, ipsilateral MBP staining was diffuse and the distinct striosomal cytoarchitecture was lost. White matter tracts were preserved in the HUCBC treated groups compared to the MCAO only groups, and were indistinguishable from the sham controls. The contralateral hemispheres of the MCAO+HUCBC and MCAO only groups resembled that of both hemispheres of the sham animal (Fig. 1A). Quantitative image analyses of MBP staining between groups showed a statistically significant reduction in discrete MBP immunofluorescence in ipsilateral striatum after MCAO alone when compared to sham - treated ($p < 0.05$, $n = 8$) and MCAO+HUCBC - treated ($p < 0.001$, $n = 8$) animals. There were no significant differences between the sham and MCAO+HUCBC groups ($p > 0.05$, $n = 8$) (Fig. 1B).

Characterization of Oligodendrocyte Culture

To examine the direct effects of HUCBCs on OL cell survival during ischemia, OLs were cultured for oxygen glucose deprivation (OGD) experiments. A battery of antibodies was first used to characterize the phenotype of the cultured cells. Antibodies specific for the O1, NG2 and MBP antigens were used to determine the stage of OL differentiation (Fig. 2), with cells expressing O1 and MBP representing a mature phenotype distinct from oligodendrocyte precursors that expressed NG2 but lacked abundant cellular processes. Approximately 60% of cultured cells expressed O1 and MBP, while approximately 90% of NG2 – positive OLs showed complex process development consistent with late stage immature OLs. This immunohistochemical profile indicates that the phenotype observed in culture is similar to that which predominates in the adult rat brain (Sommer and Schachner 1982). CNPase, an antigen expressed by both mature OLs and OL progenitors, was used to identify OLs in all subsequent cultures. Contaminating microglia were detected using anti-OX-42, an antibody that detects the CD11b receptor expressed on microglia cell surfaces (Lee et al. 2002). Prior to each OGD experiment, a coverslip was stained for both CNPase and CD11b to verify the purity of the culture. Only cultures with less than 5% microglia contamination, as measured by the percent of CD11b+ cells to DAPI+ cells, were used.

HUCBCs Reduce the Number of Caspase 3 - Positive OLs *In Vitro*

Previous studies have shown that caspase activation following OGD is a good indicator of apoptotic OLs (Shibata et al. 2000). A previously characterized antibody recognizing activated caspase-3 was used to determine as an indicator of apoptotic cells (Coirault et al. 2007; Li et al. 2008). OGD greatly increased the amount of caspase - positive OLs compared to control samples (Fig. 3A). OLs co-cultured with 10⁴ HUCBCs using transwell inserts showed a marked reduction in caspase 3 - positive cells compared to the OGD condition lacking HUCBCs. Quantification of caspase 3 - positive OLs was performed across all experimental conditions (Fig. 3B). OGD alone treatment significantly increased caspase immunoreactivity when compared to the normoxia treatment group ($p < 0.001$, $n = 3$). Co-culture of OLs with 10⁴ HUCBCs significantly reduced the percentage of caspase 3 - positive cells compared to the OGD alone treatment group ($p < 0.001$, $n = 3$). Furthermore, there was no significant difference between the normoxia groups and the OGD+HUCBC group ($p > 0.05$, $n = 3$). These data suggest that co-culture with 10⁴ HUCBCs reduces caspase 3 activity to control levels.

HUCBCs Promote Oligodendrocyte Cell Survival *In Vitro*

In order to confirm the apoptotic cell death demonstrated with the caspase immunostaining, lactate dehydrogenase (LDH) levels in the media were monitored. LDH is released from cells when membrane integrity is lost, and therefore was used as another measure of OL cell death. OL cultures that underwent OGD showed a significant increase in LDH release

compared to normoxia controls ($p < 0.001$, $n=3$) (Fig. 4). Cultures treated with 10^4 HUCBCs showed a significant reduction in the amount of LDH released relative to OGD alone ($p < 0.001$, $n=3$). The relative absorbances obtained from the LDH assays were converted to actual OL cell numbers using the standard curve as described previously. For the hypoxic groups, cells co-cultured with 10^4 HUCBCs showed a near 60% reduction in the number of dead OLs compared to those that were not treated with HUCBCs.

Discussion

Oligodendrocytes provide the framework for efficient cognitive and motor functioning by virtue of axon myelination throughout the central nervous system. During an ischemic event, OLs have been shown to be the most vulnerable of all the glia (Lyons and Kettenmann 1998). Recent reports have demonstrated white matter damage in both neonatal (Kaur et al. 2006) and adult (Gresle et al. 2006) ischemia models. Here, we show that treatment with HUCBCs was protective both *in vitro* and *in vivo*. Infusion of 10^6 HUCBCs into rats that were subjected to MCAO resulted in retention of discrete MBP - positive striosomes in corpus striatum that were greatly degraded in vehicle - treated animals after MCAO. To our knowledge, this is the first report showing that HUCBC administration can preserve white matter structures following an ischemic insult. Moreover, this cellular therapy is the first treatment which successfully accomplishes this feat at such a delayed time point.

In the developing brain, white matter injury after hypoxia-ischemia (H-I) has been linked to the inherent susceptibility of pre-OLs, indicating that this phenotype has a unique response to insult that is not observed at other developmental stages. In the adult brain, however, OL progenitors do not predominate. Instead, ischemic injury after stroke affects a fully myelinated system composed mainly of mature OLs. Previous data from OL cultures and whole tissue sections showed that the O1 antigen is expressed on OLs that exhibit a more developed phenotype than that of OL progenitors (Back et al. 2002; Craig et al. 2003). Here, a large population of cells expressed the O1 cell surface antigen. The NG2 proteoglycan was expressed ubiquitously on cellular processes but not in differentiated cells that expressed the MBP or O1 antigens, consistent with previous data identifying NG2 as a pre-OL marker (Reynolds and Hardy 1997). Importantly, the vast majority of cells that expressed NG2 in the absence of O1 or MBP displayed morphology that is consistent with late stage, immature OLs, exhibiting abundant process development. This phenotype represents a late stage of differentiation that occurs prior to development into the mature, myelinating OL. Likewise, few cells exhibited the simple bipolar morphology that is characteristic of OL progenitors. Therefore, the relatively high expression of cell surface antigens associated with differentiated OLs, coupled with the complex process development exhibited by nearly all NG2 - positive cells, indicates that these cultures were predominantly composed of late stage immature OLs and mature, myelinating OLs, both of which are abundant in the adult brain.

In vitro experiments shown here demonstrated that exposure to 2 hrs of OGD resulted in OL cell death and increased numbers of active caspase 3 immunoreactive cells when compared to cells treated with HUCBCs. These data are consistent with previous findings *in vivo* that demonstrated a role for apoptotic machinery, particularly through activation of caspase 3, in mediating the transient injury response to ischemic insult in white matter (Shibata et al. 2000). Our laboratory has shown that HUCBC treatment reverses apoptotic processes 48 hours post-MCAO (Newcomb et al. 2006). To test whether the protection afforded by HUCBCs was due to either indirect or direct actions on OLs, 10^4 HUCBCs were added to transwells in OL primary cultures prior to OGD. OGD-induced cell death was significantly reduced after treatment with HUCBCs, an effect that was associated with reduced caspase 3 - positive cells and decreased release of LDH. This suggests that HUCBC treatment may

protect OLs by secreting soluble factors. One candidate class of compounds could be growth factors such as nerve growth factor and vascular endothelial growth factor, which have previously been shown to be expressed by HUCBCs (Bracci-Laudiero et al. 2003; Ratajczak et al. 1998).

Results from the *in vivo* experiments showed marked retention of striosomal bundles within the corpus striatum of animals subjected to MCAO and treated with HUCBCs compared to saline-treated controls. Previous data indicates that the reductions in infarct volume by HUCBCs *in vivo* are likely achieved through interactions with the immune system (Newman et al. 2006; Vendrame et al. 2005). In particular, removal of the spleen significantly reduces infarct volume (Ajmo et al. In press) and HUCBC migrate to the spleen reducing its inflammatory level (Vendrame et al. 2006). Thus, peripheral immune could be detrimental not only to neurons but also to oligodendrocytes. This is consistent with the data presented here, particularly since treatment did not begin until 48 hours after MCAO surgery. It is also likely that HUCBCs are secreting factors which have direct effects on OLs to increase survival. Due to this early protection, the degree of inflammation that occurs over time in response to increased cell death would also be reduced. The *in vitro* data from primary OL cultures support this hypothesis, since protection by HUCBCs occurred in the absence of immune cells that have been shown to be primary sources of pro-inflammatory molecules in brain.

In the developing brain, H-I insults produce several discrete forms of white matter pathology that have been broadly classified as periventricular leukomalacia, often leading to severe motor deficits such as cerebral palsy (Follett et al. 2000). Both clinical and rodent studies suggest that perturbations in developing white matter result, in large part, from the death of pre-myelinating oligodendrocytes that exhibit a selective vulnerability to H-I (Back et al. 2002). In the adult brain, myelination has already taken place, and yet white matter is also compromised after ischemia. Because apoptotic machinery is also activated after ischemia in the neonate, it is possible that similar cellular mechanisms are at work in both models.

Taken together, these data provide evidence that HUCBC treatment is efficacious in reducing white matter injury in the MCAO model of embolic stroke and additionally point to a direct mechanism of action on OLs that may extend this therapeutic potential to neonatal H-I models as well. Future *in vivo* studies examining the time course of cell death and immune cell recruitment, as well as *in vitro* studies characterizing the soluble factors secreted by HUCBCs, would help to elucidate the mechanisms by which these cells afford protection to OLs after ischemia. Moreover, this treatment would be of great benefit to humans because of the greater proportion of white matter in the human brain relative to the rat (Hagberg et al. 2002).

Acknowledgments

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Abbreviations

CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
HUCBC	Human umbilical cord blood cell
LDH	Lactate dehydrogenase
MCAO	Middle cerebral artery occlusion
MBP	Myelin basic protein

OL	Oligodendrocyte
OGD	Oxygen glucose deprivation
H-I	Hypoxia-ischemia

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Figure 1. Human umbilical cord blood (HUCBC) treatment reduces middle cerebral artery occlusion (MCAO)-induced myelin basic protein (MBP) degradation

A, Photomicrographs depicting striatal brain sections stained for myelin basic protein. Loss of structural integrity and diffuse staining is evident in the ipsilateral hemisphere after MCAO, indicating white matter damage. All other sections retained discrete striosomal structure indicative of white matter retention. Scale bars represent 100 μ m. B, Quantification of MBP staining. MCAO significantly reduced the area of discrete MBP immunoreactivity when compared to sham treated animals ($p < 0.05$, $n = 8$) (asterisk). There was a significant increase in MBP immunoreactivity observed after treatment with HUCBC when compared to MCAO only treated animals ($p < 0.001$, $n = 8$) (pound sign). There was no significant difference in MBP immunoreactivity observed between the sham operated animals and the MCAO+HUCBC treated animals ($p > 0.05$, $n = 8$).

Figure 2. Cultured oligodendrocytes exhibit a mature phenotype

A, Photomicrographs depicting cultured oligodendrocytes stained for MBP (green), NG2 (red) and DAPI (blue). B, Photomicrographs depicting cultured oligodendrocytes stained for O1 (green), NG2 (red) and DAPI (blue). Scale bars represent 20 μm .

Figure 3. HUCBC treatment reduces oxygen glucose deprivation (OGD) induced Caspase 3 activation

A, Photomicrographs depicting cultured oligodendrocytes stained for CNPase (green) and active caspase 3 (red). OGD treatment alone increased the number of oligodendrocytes labeled with active caspase 3. Treatment with HUCBCs reduced the prevalence of active caspase 3 staining similar to control. Scale bars represent 100µm. B, Quantification of active caspase 3 staining. OGD treatment significantly increased active caspase 3 immunoreactivity ($p<0.001$, $n=3$), while treatment with HUCBCs significantly reduced the percentage of caspase 3-positive cells ($p<0.001$, $n=3$). Asterisk indicates significance at $p<0.001$

Figure 4.

HUCBC treatment suppresses OGD induced Lactate dehydrogenase (LDH) release. Graph depicting LDH release from cultured oligodendrocytes. Oligodendrocyte equivalents were determined by generating a standard curve based on LDH released from known numbers of oligodendrocytes lysed with Triton X-100. OGD significantly increased the number of ruptured oligodendrocytes ($p < 0.001$, $n = 3$), while HUCBC treatment significantly reduced this effect ($p < 0.001$, $n = 3$) such that LDH levels did not differ from normoxia controls ($p > 0.05$, $n = 3$). Asterisk indicates significance at $p < 0.001$.