STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

IFATS Collection: The Conditioned Media of Adipose Stromal Cells Protect Against Hypoxia-Ischemia-Induced Brain Damage in Neonatal Rats

XING WEI,[a] ZHIMEI DU,[b] LIMING ZHAO,[a] DONGNI FENG,[c,d] GANG WEI,[a] YONGZHENG HE,[a] JIANGNING TAN,[a] WEI-HUI LEE,[e] HARALD HAMPEL,[f] RICHARD DODEL,[g] BRIAN H. JOHNSTONE,[c,d] KEITH L. MARCH,[c,d] MARTIN R. FARLOW,[a] YANSHENG DU[a,b,g]

Departments of [a]Neurology, School of Medicine, [c]Medicine, and [e]Pediatrics, [d]Indiana Center for Vascular Biology and Medicine, and [h]Indiana University Center for Aging Research, School of Medicine, Indiana University, Indianapolis, Indiana, USA; [b]Department of Biochemistry and Molecular Biology Laboratory, The Rockefeller University, New York, New York, USA; [f]Discipline of Psychiatry, The Adelaide & Meath Hospital Incorporating the National Children's Hospital, Trinity College, University of Dublin, Dublin, Ireland; [g]Department of Neurology, Philipps University, Marburg, Germany

Key Words. Adipose stromal cells • Hypoxia-ischemia • Growth factor • Rat

ABSTRACT

Adipose tissue stroma contains a population of mesenchymal stem cells, which support repair when administered to damaged tissues, in large part through secreted trophic factors. We directly tested the ability of media collected from cultured adipose-derived stem cells (ASCs) to protect neurons in a rat model of brain hypoxic-ischemic (HI) injury. Concentrated conditioned medium from cultured rat ASCs (ASC-CM) or control medium was infused through the jugular vein of neonatal Sprague-Dawley rats subjected to HI injury. The ASC-CM was administered either 1 hour before or 24 hours after induction of injury. Analysis at 1 week indicated that administration at both time points significantly protected against hippocampal and cortical volume loss. Analysis of parallel groups for behavioral and learning changes at 2 months postischemia demonstrated that both treated groups performed significantly better than the controls in Morris water maze functional tests. Subsequent post-mortem evaluation of brain damage at the 2-month time point confirmed neuronal loss to be similar to that observed at 1 week for all groups. We have identified several neurotrophic factors in ASC-CM, particularly insulin-like growth factor-1 and brain-derived neurotrophic factor, which are important factors that could contribute to the protective effects of ASCs observed in studies with both in vitro and in vivo neuronal injury models. These data suggest that delivery of the milieu of factors secreted by ASCs may be a viable therapeutic option for treatment of HI, as well as other brain injuries. STEM CELLS *2009;27:478–488*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The recent discovery of a population of pluripotent cells in adipose tissue has revealed a novel source of cells with potential uses for autologous cell therapy to regenerate damaged or diseased tissues [1, 2]. The pluripotent cells reside in the "stromal" or "nonadipocyte" fraction of the adipose tissue and were previously considered to be preadipocytes; however, more recently, adipose-derived stem cells (ASCs) from mice, rats, nonhuman primates, and humans were demonstrated to exhibit differentiation into neural and glial cells in vivo and in vitro [1, 3–11]. When exposed to neuronal induction media, mouse ASCs expressed voltage-dependent calcium channels, as well as low levels of nestin and synaptin I, both markers of differentiated neurons [4]. In a rat middle cerebral artery occlusion model of ischemic brain injury, transplanted predifferentiated human ASCs migrated to areas of ischemic injury and expressed neuronal-specific markers in conjunction with functional benefit [9].

Despite the improvements observed, there is no conclusive evidence from these previous studies that human ASCs truly differentiated in vivo into functional neurons that formed connections with rat neurons [12], and it is possible that functional improvement of these animals may be, in part, due to trophic support provided to host cells from factors released by ASCs, such as we have observed in the repair of acute ischemic

Author contributions: X.W.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; Z.D., H.H., and R.D.: data analysis and interpretation, manuscript writing; L.Z., D.F., G.W., Y.H., J.T.: perform experiments; W.-H.L.: design of behavior tests; B.H.J.: provision of study material or patients, manuscript writing, data analysis and interpretation; K.L.M.: provision of study material or patients, manuscript writing, data analysis and interpretation, financial support; M.R.F.: administrative support; Y.D.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

Correspondence: Yansheng Du, Ph.D., Department of Neurology, School of Medicine, Indiana University, 975 West Walnut Street IB457, Indianapolis, Indiana 46202, USA. Telephone: 317-277-2659; Fax: 317-277-6146; e-mail: ydu@iupui.edu Received April 2, 2008; accepted for publication November 6, 2008; first published online in STEM CELLS *EXPRESS* November 20, 2008. 1066-5099/2009/$30.00/0 doi: 10.1634/stemcells.2008-0333

damage in skeletal muscles [13, 14]. ASCs secrete several potentially beneficial growth factors, such as granulocyte-macrophage colony-stimulating factor, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor, transforming growth factor-β, and insulin-like growth factor-1 (IGF-1) [14–16]; these may protect neurons from injury, as well as promoting endogenous repair. Neurotrophic factors, such as IGF-1 and erythropoietin have been shown to rescue different types of neurons from ischemia, hypoxic-ischemic (HI) injury, neurotoxins, and excitotoxicity [17–24].

HI encephalopathy (HIE) during the prenatal and perinatal period is a major cause of damage to the fetal and neonatal brain, resulting in considerable incidence of morbidity and mortality [25]. Currently, there is a paucity of effective treatment options for limiting the consequences of neonatal HI. As a result, between 20% and 30% of affected infants will die during the newborn period, whereas among the survivors, up to 25% exhibit lifelong motor and/or mental deficits [26–29]. Therapeutic ASCs present an opportunity for developing treatments that will reverse or prevent the damaging effects of HI injury. However, an important challenge for translation to the clinic lies in development of safe and effective delivery methods for cells that are minimally invasive and free of secondary complications and that ensure that cells are distributed throughout injured tissues to maximize the effect while alleviating the potential for untoward effects in nontarget tissues [30]. In addition, the brain is a privileged organ that may be refractory to targeting of cell-sized agents delivered through the peripheral system; thus, bypassing the blood-brain barrier requires invasive delivery methods. Since there is ample evidence that the beneficial effects of stem cells may be conferred predominantly indirectly through paracrine mechanisms, rather than direct tissue regeneration [13, 31–34], we designed this study to evaluate whether factors secreted by ASCs during culture are sufficient to potently protect against brain damage when delivered through the peripheral venous system, both preceding and following injury. In particular brain lesions resulting from HI injury in neonates is associated with significant long-term spatial memory deficits in rats [22]. We used the commonly used Morris water maze test [35] to evaluate whether the structural preservation seen in association with conditioned medium from cultured rat ASCs (ASC-CM) was accompanied by a long-term functional benefit with respect to memory deficits. Furthermore, primary cultured cerebellar granular neurons (CGN), which are widely used for neuronal mechanistic studies as well as apoptosis and neural injury models, were used to determine the specific contributions of IGF-1 and brain-derived neurotrophic factor (BDNF) in ASC-CM to neuroprotection [36, 37]. Finally, we confirmed in vivo that both IGF-1 and BDNF in ASC-CM are essential for recovery from neuropathological injury and markedly reduce long-term functional cognitive and motor skill impairments in a rat model of HI injury.

MATERIALS AND METHODS

Isolation of Rat and Human ASCs

Rat subcutaneous adipose tissue samples were obtained from inguinal and perirenal fat pads of animals killed by CO_2 asphyxiation. Adipose tissues were minced before digesting in 1 mg/ml Collagenase Type I solution (catalog no. 4196; Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) under gentle agitation for 1 hour at 37°C. The digested mixture was diluted with 50 ml of culture medium (Dulbecco's modified Eagle's medium [DMEM], high glucose, 10% fetal bovine serum [FBS]) and then centrifuged at 200*g* for 5 minutes to separate the stromal cell fraction (pellet) from adipocytes. The supernatant was removed, and the cellular pellet was resuspended in 20 ml of fresh medium (DMEM/10% FBS), and then serially filtered through 250-μm Nitex 03–250/50 cloth (Sefar American Inc., Kansas City, MO, http://www.sefar.com) and a 100-μm cell strainer (BD Biosciences, San Diego, http://www.bdbiosciences.com) to remove debris. The filtrate was again centrifuged at 200*g* for 5 minutes. The pellet containing ASCs was treated with red blood cell lysis buffer for 5 minutes at 37°C and then pelleted at 300*g* for 5 minutes. The cell pellets were resuspended in Endothelial Growth Medium 2-MicroVascular (EGM2-MV; Lonza, Walkersville, MD, http://www.lonza.com). ASCs were plated in an uncoated T75 tissue culture flask at a density of 4×10^6 cells per cm^2 and incubated in a humidified chamber at 37° in an atmosphere of 5% CO_2. After overnight culture, the medium was replaced with EGM2-MV (Lonza).

Collection and Concentration of ASC-CM

Rat ASCs were cultured to confluence in 100-mm culture dishes containing EGM2-MV, and each dish was rinsed and replenished with 5 ml of Basal Media Eagle (BME) containing 5 mM KCl; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 24 hours. Media were collected and then concentrated by a factor of 250× and desalted by centrifugation at 3,000*g* using CentriPlus centrifugal filters (Millipore Corporation, Bedford, MA, http://www.millipore.com) (molecular weight cutoff, 10,000).

Animals and Treatment

Animal studies were approved by the Indiana University School of Medicine Institutional Animal Use and Care Committee. Pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, http://www.criver.com) were individually housed in cages and fed standard laboratory chow ad libitum. All animal experiments and analyses were grouped randomly and performed blindly with respect to treatment. Hypoxia-ischemia was induced in 7-day-old rat pups as previously described [25, 38] with minor modifications. Briefly, 7-day-old pups (eight per group, weighing 18–19 g) were anesthetized with a mixture of isoflurane (3% for induction, 1.5% for maintenance) and 30% oxygen/70% N_2. The left common carotid artery of each pup was exposed, isolated from the nerve and vein, and ligated with 3-0 surgical silk. Following ligation, the wound was sutured, and the rat pups were returned to their dams for 3 hours to allow for recovery. Sham-operated animals underwent the same operative procedure except that the exposed carotid artery was not ligated (global hypoxia only). Thereafter, all pups were placed in 2-l airtight and watertight jars and exposed to a humidified nitrogen-oxygen mixture (8% oxygen) delivered at 5–6 l per minute. During the 150 minutes of hypoxia, the jars were partially submerged in a 37°C water bath to maintain a constant thermal environment. Pups were then returned to their dams and housed under a 12-hour light/dark cycle with food and water until they were sacrificed. Approximately 10% of the animals died during the HI treatments and were excluded from the study.

Pups received jugular vein injections of 10 μl of 250-fold concentrated ASC-CM or BME at 1 hour before or at 24 hours after the HI injury (re-surgery). The contribution of BDNF and IGF-1 to the observed neuroprotective effect was assessed after preincubating ASC-CM for 30 minutes with neutralizing antibodies to BDNF (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and IGF-1 (Millipore) or, as a control, an isotypic antibody. For administration, animals were anesthetized with isoflurane before a small incision was made on the right side of the neck to expose the right external jugular vein after gently dissecting down through the salivary and lymphoid glands, adipose tissue, and fascia. The vein was gently elevated and cleaned before insertion of a 25-gauge needle affixed to a 0.3 cc tuberculin syringe for injection. All groups of animals in each experiment were treated and analyzed concurrently to eliminate intergroup variation.

Tissue Preparation for Assessment of Brain Infarct Volume and ASC-CM Penetration into the Brain Following i.v. Delivery

On postnatal days 14 (P14) or 91 (P91), rats were deeply anesthetized with 0.2 mg/g of body weight methohexital by intraperitoneal

injection and perfused with 5 ml of isotonic saline by transcardiac puncture, followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Coronal sections from the genu of the corpus callosum to the end of the dorsal hippocampus were stained with cresyl violet as described previously [25, 39]. To determine infarct volume, the cross-sectional areas of the striatum, hippocampus, cortex, and thalamus in each of eight equally spaced reference planes were photomicrographed and the area of infarct for each brain region was analyzed using SPOT software (Diagnostic Instrument, VA, http://www.diaginc.com). The sections used for quantification corresponded approximately to plates 12, 15, 17, 20, 23, 28, 31, and 34 in the rat brain atlas [25, 39, 40]. The thickness of the reference planes was estimated by recording the number of 50-μm sections required to section to the rostral aspect of the hippocampus. The volume of the hippocampus, cortex, thalamus, and striatum was then estimated [41]. The percentage volume occupied by HI-induced lesion in the different areas was obtained by dividing the sum of the damaged areas ipsilateral to the carotid ligation (left side) by the sum of the ipsilateral areas of the corresponding uninjured, normal area (right side).

ASC proteins were labeled with a biotin protein labeling kit (catalog no. 11418165001; Roche Applied Science, Indianapolis, IN, http://www.roche.com/diagnostics) according to the manufacturer's instructions. In brief, free amino groups of the ASC proteins were coupled to D-biotinoyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (biotin-7-NHS). Nonreacted biotin-7-NHS was separated on a Sephadex G-25 column (Roche Applied Science). Under anesthesia (isoflurane), the biotinylated ASC proteins were injected directly into the jugular vein of P7 rat pups (20 μg in 40 μl for each rat) at 1 hour before or 24 hours after HI. Under anesthesia (100 mg/kg ketamine and 20 mg/kg xylazine), the cerebrospinal fluid (CSF) was needle-aspirated at 72 hours after hypoxia-ischemia from the fourth cerebral ventricle. Briefly, rats were anesthetized and the head was fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, http://www.kopfinstruments.com). The atlanto-occipital membrane was exposed, and a cannula with a 26-gauge needle tip attached was inserted into the cisterna magna. Freely flowing CSF was collected into ice-cold polypropylene tubes. CSF samples found to be free of blood, as determined by spectrophotometric analysis for hemoglobin, were stored at −20°C until being assayed [42]. The concentrations of biotinylated proteins in CSF and serum were measured using Reacti-Bind Streptavidin High Binding Capacity Coated Plates (catalog no. 15502; Pierce, Rockford, IL, http://www.piercenet.com). Each value is expressed as mean ± SEM.

After the CSF was obtained, brains were removed and sectioned. Fresh frozen sections were fixed in 4% paraformaldehyde and then washed with phosphate-buffered saline + 0.05% Tween-20. Fluorescent avidin (Alexa Fluor 488; catalog no. 21370; Invitrogen) was added to tissue sections. The distribution of biotinylated ASC-CM proteins was visualized under a microscope.

Morris Water Maze

The maze consists of a light blue circular pool, 1.8 m in diameter and 0.6 m in height, filled to a level of 35 cm with water maintained at a constant temperature of 27°C [35]. Pool water was made opaque by adding 150 ml of nontoxic white tempera paint. A Plexiglas escape platform (20 cm in diameter) was positioned 1 cm below the water surface at various locations throughout the pool. The platform was retracted manually during probe trials. A circular white curtain surrounded the pool, extending from ceiling to floor. Three types of trials (visible, invisible, and probe) were performed. Three types of evaluations were each performed three times in succession to determine learning/memory. The first assessment was a visible platform test with a flag on the submerged platform to provide a visual cue to the animal. The rats were scored for the time taken to find the platform and exit the water (termed latency period). The second test was an invisible platform test, which evaluates spatial memory, where the rat is required to locate the platform based on memory attained through the previous tests using the aid of the visual cue; this invisible platform test was performed immediately after the visible platform experiment. The final assessment, termed a probe trial, is a measure of both cognitive reasoning and memory, where, immediately after the invisible platform tests were performed, the platform was removed and the maze test was again performed. The rat is assessed for the amount of time spent searching in the quadrant previously containing the platform.

For the first trial, which was conducted on day 67 following induction of HI, rats were trained and evaluated using the following experimental paradigms. First, in the visible platform experiment, the platform was placed in the second quadrant with a flag placed in the center to provide an external visual maze cue. Rats were gently placed in the pool at the fourth quadrant facing the pool wall. The time that it took for rats to find the platform was recorded by an observer. Afterward, rats were allowed to rest on the platform for 30 seconds before the trial was repeated. In the event that a rat could not locate the platform in 60 seconds, it was manually placed on the platform (in which case 60 seconds was recorded as the time). Three independent trials for each test were performed per rat. The second test was an invisible platform experiment to assess spatial memory. Immediately after the tests were conducted with the visible platform, the flag was removed and the trials were repeated as described. Three independent trials of this test were performed. A probe test is a further cognitive measure for working memory. At the end of the sixth day of testing and 1 hour after the final platform trial, each rat received a 1-minute probe test, which involved placing the rats in the pool, as above, except that no platform was present. The amount of time spent in the quadrant that previously contained the platform was recorded. All three tests were performed on each of 3 days (specifically days 67, 68, and 69).

Enzyme-Linked Immunosorbent Assay

Levels of BDNF and IGF-1 in ASCs were measured using an enzyme-linked immunosorbent assay (ELISA) kit (CYT306 for BDNF [Chemicon, Temecula, CA, http://www.chemicon.com] and AC-18F1 for IGF-1 [Immunodiagnostic Systems Inc., Fountain Hills, AZ, http://www.idsplc.com]) according to the manufacturers' instructions. All samples and standards were measured in duplicate.

Western Blot Analysis

Proteins were extracted from rat brains using cold lysis buffer (10 mM tetrasodium pyrophosphate, 20 mM Hepes, 1% Triton X-100, 100 mM NaCl, 2 μg/ml protinin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride). Protein concentrations from tissue extracts or ASC-conditioned medium were determined using the Bradford protein assay. Equal amounts of protein were placed in 2× sample buffer (0.125 M Tris-HCl, pH 6.8, 2% glycerol, 0.2 mg/ml bromphenol blue dye, 2% SDS, and 10% β-mercaptoethanol) and electrophoresed on 10% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane by electroblotting. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with Tween-20 (TBST) and 5% nonfat milk. Primary antibodies (1:1,000) at the appropriate dilution were incubated for 1 hour at room temperature in TBST and 5% nonfat milk. Blots were then washed and incubated with a peroxidase-conjugated secondary antibody (1:2,000) for 1 hour in TBST. The chemiluminescent substrate for the secondary antibody was developed with the ECL detection system (GE Healthcare, Piscataway, NJ, http://www4.gelifesciences.com). Blots were exposed to film for 3–5 minutes and developed.

Primary Cerebellar Granular Neuron Cultures and Neurotoxicity Assays

CGN were prepared from 8-day-old rat as previously described [43]. Briefly, freshly dissected cerebella were dissociated, and the cells were seeded at a density of 1.2–1.5 × 10^6 cells per milliliter on poly-L-lysine-coated dishes in BME supplemented with 10% FBS (Invitrogen), 25 mM KCl, and gentamicin (0.1 mg/ml; Invitrogen). Cytosine arabinoside (10 μM; Sigma-Aldrich) was added to the culture medium at 24 hours after initial plating. All experiments used neurons after 7–8 days in vitro. The glutamate treatments followed methods that have been previously described [43–46]. The model of CGN apoptosis by serum and K^+ deprivation involved culturing isolated CGN in BME then switching to BME containing 5 mM K^+ (low K). After 24 hours, medium in some wells was

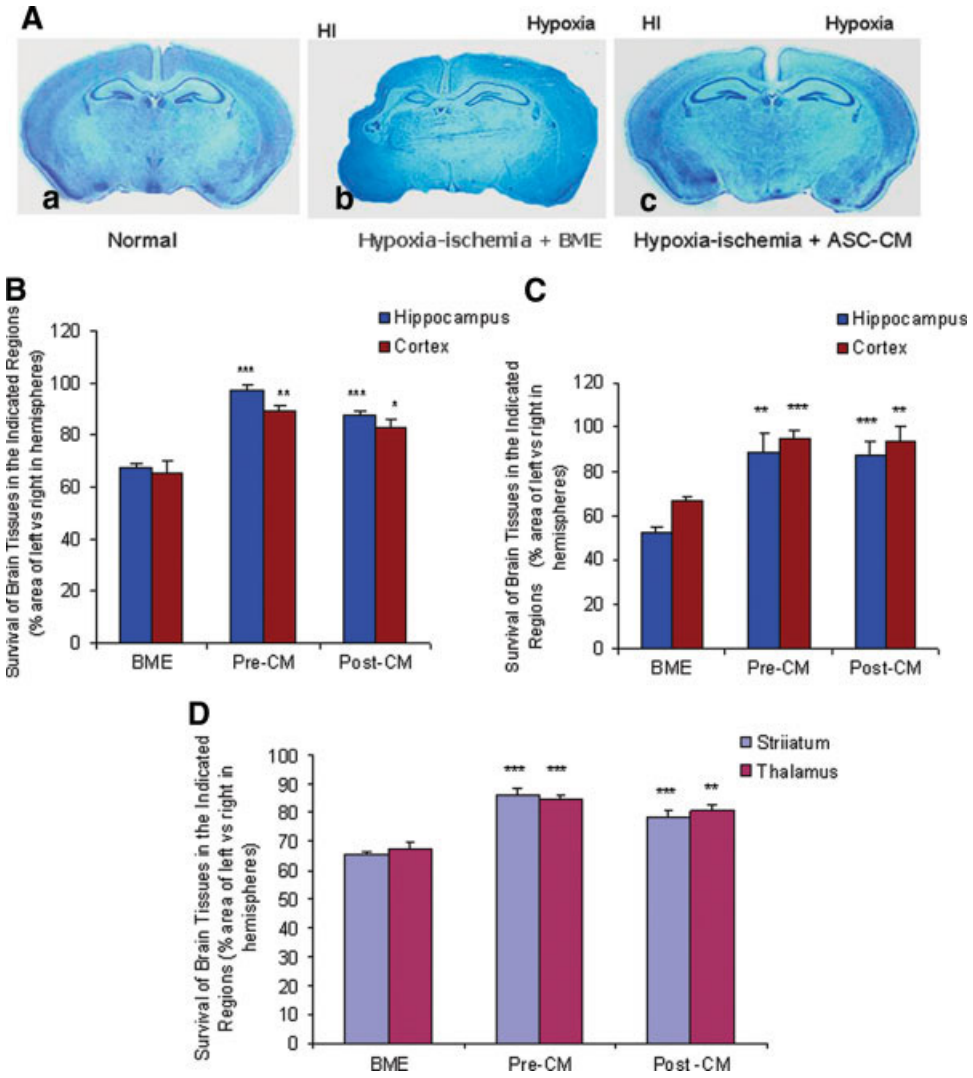

Figure 1. ASC-CM prevented brain tissue loss following hypoxic-ischemic injury. **(A):** Representative coronal sections of postnatal day 14-day rat brains demonstrating morphology of normal brains **(Aa)** and brains subjected to HI (left hemisphere) or hypoxia only (right hemisphere) after i.v. infusion of BME **(Ab)** or ASC-CM **(Ac)**. Rats subjected to HI received either BME **(Ab)** or ASC-CM intravenously at 24 hours following hypoxia treatment. **(B–D):** Brains were stained and analyzed histologically as described in Materials and Methods. Animals were all subjected to HI at day 7, and analysis was performed at days 14 **(B)** and 91 **(C, D)**. Control animals were given i.v. infusions of BME 1 hour before HI. The other animals in the study were infused with ASC-CM either 1 hour before (pre-CM) or 24 hours after (post-CM) being subjected to HI. The area of tissue in the lesioned hippocampal, cortical, thalamic, or striatal hemispheres of each animal was compared with the ipsilateral hemisphere and reported as a ratio (mean ± SEM). *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: ASC-CM, conditioned medium from cultured rat adipose-derived stem cells; BME, Basal Media Eagle; CM, conditioned medium; HI, hypoxia-ischemia.

replaced with the same volume of BME (low K). Viable neurons were quantified by counting fluorescein (green)-positive cells, which result from the de-esterification of fluorescein diacetate (FDA; Sigma-Aldrich) by living cells. Briefly, cultures were incubated with FDA (10 μg/ml) for 5 minutes, examined, and photographed using UV light microscopy, and the number of neurons from representative low power fields was counted as previously described [43]. Propidium iodide (PI; Sigma-Aldrich), which interacts with nuclear DNA of nonviable cells, producing a red fluorescence, was used to identify dead neurons. For PI staining, cultures were incubated with PI (5 μg/ml), examined and photographed using UV light microscopy as previously described [43].

Statistical Analysis

Unless indicated otherwise, data are given as means ± SEM; with the number of determinations (n) representing separate experiments carried out independently using single or duplicate samples. Data were evaluated using one-way analysis of variance, and a p value of less than .05 was considered significant.

RESULTS

ASC-CM Markedly Attenuated Both Short-Term and Long-Term Effects of HI-Induced Brain Damage

To investigate the neuroprotective effects of ASC-CM on HI-induced neuronal death in vivo, 7-day-old rats were given a single infusion of BME or ASC-CM at 1 hour before or either 24 or 72 hours after induction of unilateral HI injury. Brain tissue atrophy was initially assessed 7 days after HI injury by morphometric quantitation of the cortical, hippocampal, striatum, and thalamic regions in lesioned and healthy ipsilateral

hemispheres [39]. There was no difference in the size of lesions between BME groups treated at the two time points; therefore, for clarity, only the data from the group treated at 1 hour before injury are reported. Loss of tissue in the cortical and hippocampal regions of the HI-lesioned hemisphere was 32.8% ± 1.7% and 34.6% ± 4.5% ($n = 7$), respectively, of the ipsilateral uninjured hemisphere in animals treated with BME, indicating a significant degree of atrophy in these animals (Fig. 1A, 1B; supporting information Tables 1 and 2). Similar data were obtained in two independent repetitions (total number of animals in all experiments was 17). Loss of brain tissue was significantly reduced in rats that received 250-fold concentrated ASC-CM at 1 hour before or 24 hours after HI injury. Only 2.7% ± 1.8% of hippocampus and 11.0% ± 2.3% of cortex were lost in the group treated 1 hour before HI injury ($n = 6$; $p < .01$ and 0.001, respectively, compared with control). Similar data were obtained in subsequent repeats of this experiment (total $n = 14$). In animals treated 24 hours following injury, 12.2% ± 1.5% of hippocampal and 17.3% ± 3.3% of cortical volume was lost ($n = 6$; $p < .001$ and $p < .05$, respectively, compared with control). Similar data were obtained in subsequent repeats of this experiment (total $n = 15$). There was no measurable effect of ASC-CM when animals were treated 72 hours following HI injury (data not shown). Chronic changes in brain tissue damage were also assessed histologically at 91 days after HI injury (Fig. 1C; supporting information Tables 1 and 2). The degree of tissue loss was qualitatively similar to that observed at 7 days. Approximately 47.7% ± 2.6% and 33.0% ± 1.8% of hippocampus and cortex, respectively ($n = 6$), had atrophied in the brains of control animals. The long-term effect of ASC-CM treatment was significant reduction in the lesioned area compared with BME-treated animals ($p < .01$ in all regions for both groups compared with control). The hippocampal area of the HI-injured hemisphere in the group pretreated with ASC-CM ($n = 7$) was reduced to 11.4% ± 8.4% in comparison with control animals. Loss of cortical area in this same group was 4.7% ± 3.1%. Treatment with ASC-CM at 24 hours following HI injury ($n = 6$) limited loss to 12.3% ± 6.4% of area in the hippocampus region of the injured hemisphere, whereas there was 6.3% ± 6.7% loss in the cortex.

In addition to protection of the hippocampus and cortex, similar long-term neuroprotective effects of ASC-CM were found in areas of thalamus and striatum at 91 days after HI injury (Fig. 1D; supporting information Table 3). Approximately 32.4% ± 1.8% and 34.4% ± 0.9% of thalamus and striatum, respectively ($n = 6$), had atrophied in the brains of untreated animals. Loss of area in these regions was substantially reduced by treatment with ASC-CM. The volume of the lesion in the thalamus and striatum of the HI-injured hemisphere in the group pretreated with ASC-CM ($n = 7$) was 11.0% ± 2.3% and 14.0% ± 2.7%, respectively ($p < .001$). Treatment with ASC-CM at 24 hours following HI injury ($n = 6$) led to loss of 19.5% ± 2.2% of tissue in the thalamus of the injured hemisphere, whereas there was 21.4% ± 2.0% loss in the striatum ($p < .001$ and $p < .01$, respectively).

Protein Factors in ASC-CM Penetrate the Blood-Brain Barrier of HI-Injured Brains

It has been demonstrated previously that a temporary disruption of the blood-brain barrier (BBB) typically occurs between 24 and 72 hours after HI injury [47–50], which is likely associated with the disruption of endothelial gap junctions in resulting from the inflammatory response that occurs during this period [51, 52]. Penetration of protein components of ASC-CM into the brain was confirmed by our studies of biotinylated ASC-CM. Concentrated ASC-CM was labeled

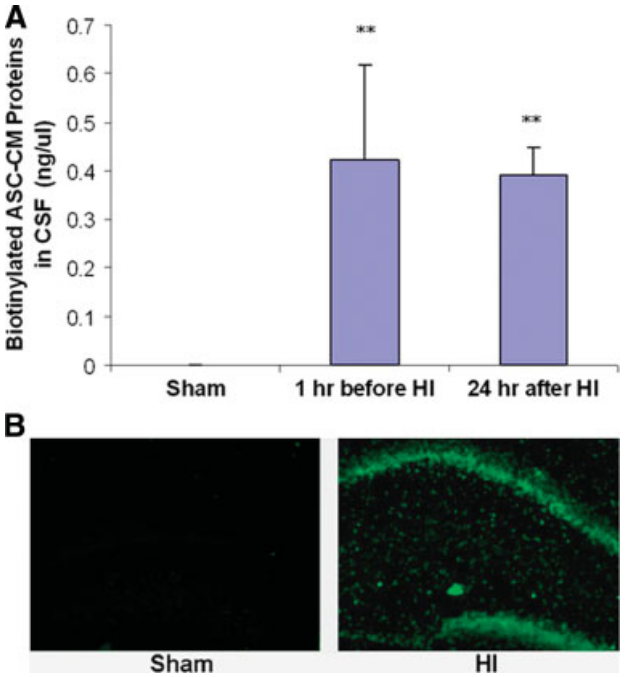

Figure 2. Factors within ASC-CM penetrated the blood-brain barrier and bound to neurons in affected regions of the brain. Concentrated ASC-CM was labeled with biotin and infused through the jugular vein either 1 hr before ($n = 3$) or 24 hrs after ($n = 4$) HI injury was induced. In addition, control animals subjected to hypoxia only were treated ($n = 9$). **(A):** After 72 hrs CSF was aspirated and the amount of biotinylated protein in the CSF was quantitated as described in Materials and Methods. **(B):** Brains were also removed, sectioned, stained with fluorescein-conjugated streptavidin, and then imaged by immunofluorescence microscopy at 1,000-fold magnification. Representative images from sections of sham- and HI-injured brains are shown. **, $p < .01$. Abbreviations: ASC-CM, conditioned medium from cultured rat adipose-derived stem cells; CSF, colony-stimulating factor; HI, hypoxia-ischemia; hr, hour.

with biotin before infusing at either 1 hour before ($n = 5$) or 24 hours after ($n = 4$) induction of HI. For comparison, sham animals which were subjected to hypoxia only were also included. The results clearly show penetration of ASC-CM into CSF of the brain, as well as predominant binding of labeled proteins to structures within the affected regions of the brain (Fig. 2). Thus, it is apparent that factors within ASC-CM delivered through the peripheral system gain entrance to the brain to act at the site of ischemic injury.

ASC-CM Attenuated the Deficit in Spatial Learning and Memory Associated with HI

Tests of spatial learning were conducted using the Morris water maze test at 2 months after HI injury, and the results are displayed in Figure 3. In all maze tests the ASC-CM-treated rats (pretreatment, $n = 7$; post-treatment, $n = 6$) performed significantly better ($p < .01$ or greater) than the BME-treated rats ($n = 6$); furthermore, the scores of the HI-injured rats treated with ASC-CM were not significantly different from those of the sham-treated (subjected to hypoxia only, $n = 6$) rats. The ASC-CM and sham groups required 8 ± 1.3 seconds to find the platform during the initial trial of the visible platform test and improved to 4 ± 0.44 seconds by the third trial (Fig. 3A). Conversely, BME-treated rats required nearly 12 ± 1.9 seconds to initially find the platform and showed significantly less learning capacity by improving to only 8.5 ± 0.55 seconds by the third attempt

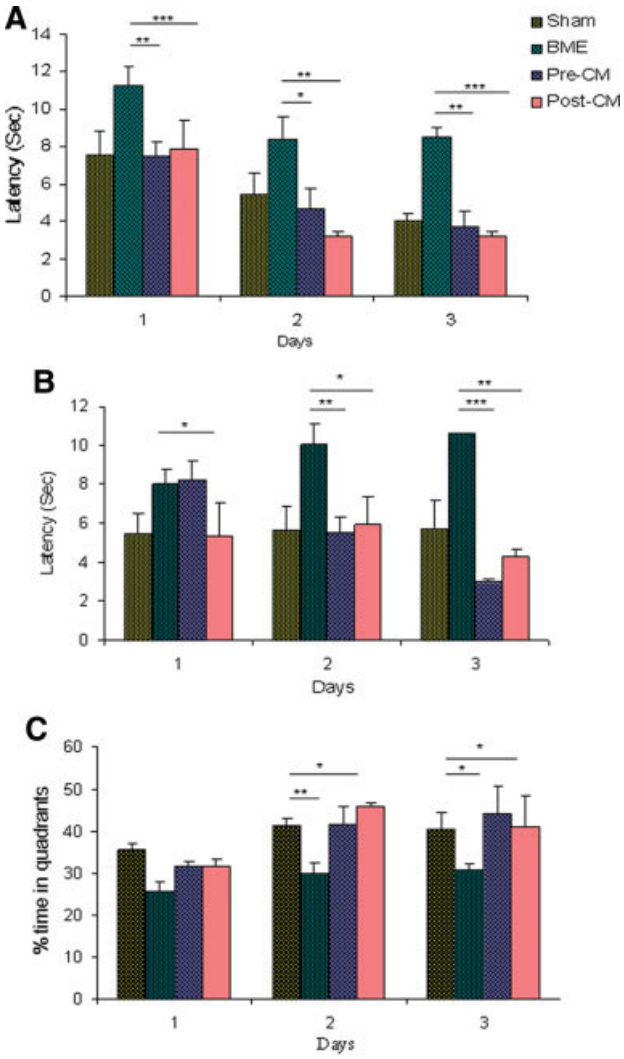

Figure 3. CM from cultured rat adipose-derived stem cells improved long-term learning and cognition. At 2 months of age each animal from the groups described in the legend to Figure 1C was evaluated for cognitive and motor skill performance using the Morris water maze test. The test was administered at 2 months of age. The data are reported as the time interval between start of the test and when the rat emerged from the water onto the platform (latency). Three different assessments were performed as described in Materials and Methods: a visible platform test **(A)**, a hidden platform test **(B)**, and a final test (conducted after the previous two) where the platform was removed **(C)**. Data are presented as mean ± SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$ compared with BME-treated animals. Abbreviations: BME, Basal Media Eagle; CM, conditioned medium; sec, second.

($p < .01$). In the submerged (invisible) platform test, BME-treated rats found the platform in 8.1 ± 0.77 seconds upon the first round of testing and took 10.8 ± 0.96 seconds to find the platform by the third test (Fig. 3B). Conversely, ASC-CM treated rats took less time to find the submerged platform initially and improved to 3.0 ± 0.13 (pretreatment) and 4.0 ± 0.33 seconds (post-treatment) by the third round of testing ($p < .01$). For comparison, sham animals took 5.8 ± 1.44 seconds to find the submerged platform on the third attempt.

In the probe test sham, ASC-CM pretreated and post-treated rats spent a similar amount of time (between 40% and 48% of their time in the pool) in the quadrant previously containing the platform, whereas BME-treated rats spent only 32% of their time in the correct quadrant (Fig. 3C). These data further demonstrated that treatment with ASC-CM improved spatial and working memory deficits at 2 months following HI.

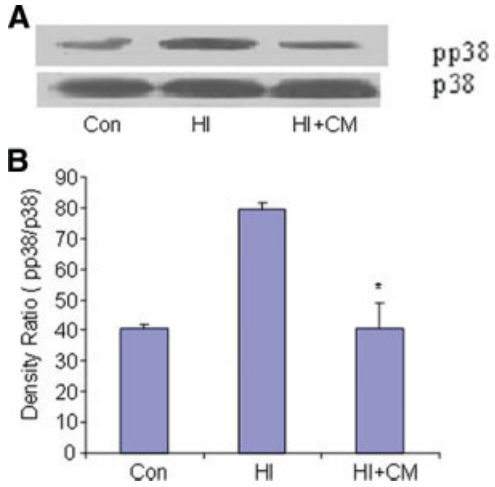

Figure 4. CM from cultured rat adipose-derived stem cells (ASC-CM) blocked HI-induced p38 activation. On day 7 after HI or ischemia only (sham control), rats ($n = 3$ per group) were killed and the hippocampus was removed and homogenized for Western blot analysis with an antibody against pp38. The density of each band detected by autoradiography (representative image shown) was determined and used to determine the ratio of pp38 to total p38. The experiment was repeated three times and reported as mean ± SEM. ASC-CM blocked the increase in pp38 after HI treatment. *, $p < .05$. Abbreviations: CM, conditioned medium; Con, control (hypoxia only); HI, hypoxic-ischemic; pp38, phosphorylated p38 protein.

One Mechanism by Which ASC-CM Protects Against HI-Induced Damage Is Blocking the Activation of p38 MAPK

Rats subjected to HI injury and treated with ASC-CM clearly showed reduced functional and morphological deficits compared with control rats. We next examined the underlying neuroprotective mechanisms conferred by ASC-CM in the neonatal HI model. Previous studies have shown that HI-induced neuronal death has features of apoptosis [53, 54] with prominent involvement of activated p38 MAPK [55]; therefore, we assayed brain tissues of HI-injured pups for levels of p38 phosphorylation to determine whether ASC-CM could block activation. An additional set of animals was used for this study, and brain tissues were harvested at 24 hours after HI injury to capture early events. A greater magnitude of protection from brain atrophy was observed in HI-injured rats pretreated with ASC-CM compared with those treated 24 hours after injury; therefore, to maximize our chances of detecting differences, only the former group was included, as well as sham and BME-treated groups for comparison ($n = 3$ each in group; all groups were treated and tissue samples were collected concurrently). The ratio of phosphorylated to unphosphorylated p38 increased by twofold in the HI-injured hemisphere of hippocampus of control rats (Fig. 4). Conversely, the level of phosphorylated (pp38) in the same region of the brain from ASC-CM-treated animals was no different from that of sham animals. Thus, ASC-CM contains an activity that blocks postischemic p38 activation.

An Important Factor in ASC-CM That Provides Protection Against Apoptosis of Primary Neurons In Vitro Is IGF-1

To further determine the mechanisms by which ASC-CM inhibits apoptosis in brain neurons, we first conducted in

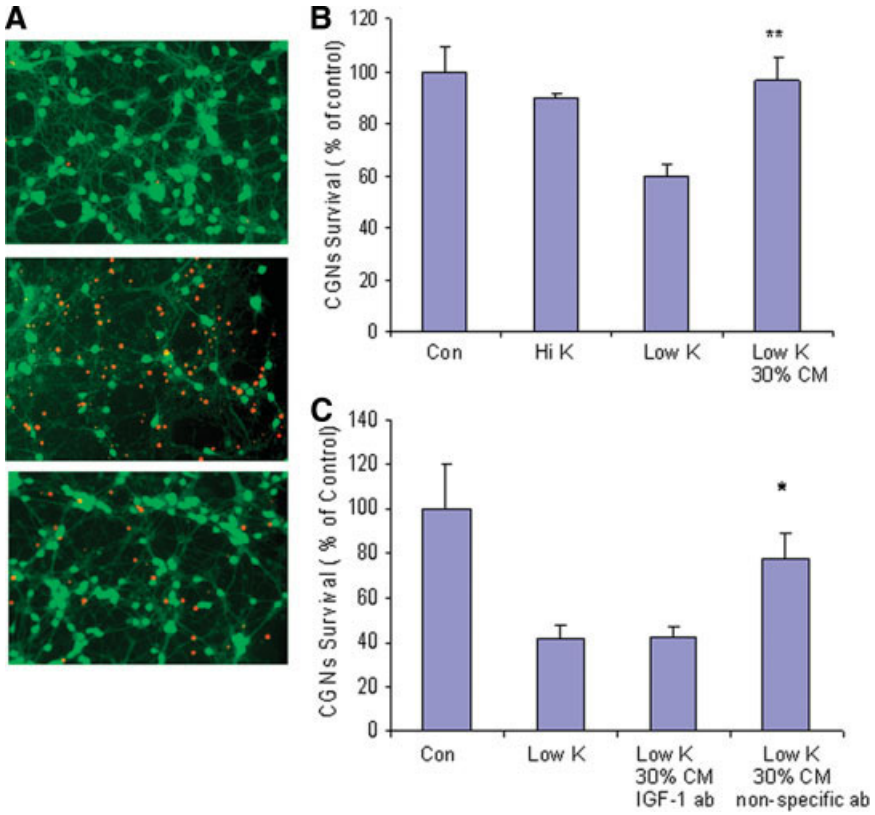

Figure 5. CM from cultured rat adipose-derived stem cells (ASC-CM) protection against serum and K^+ deprivation-induced CGN death was blocked by an IGF-1-neutralizing ab. Rat CGN were cultured in Basal Media Eagle (BME) with 25 mM K^+ and 10% serum in the presence or absence of 30% (by volume) ASC-CM (30% CM). After 24 hours the medium was replaced with Hi K or Low K, as indicated. A Con was maintained in the original BME containing serum and 25 mM K^+. **(A):** Fluorescent micrographs of neurons after treatment with fluorescein diacetate (green) to detect live cells and propidium iodide (red) to detect dead cells. Top panel, CGN grown in Hi K with serum (Con); middle panel, CGN cultured under Low K without serum for 24 hours; bottom panel, CGN cultured under Low K and without serum but with the addition of 30% ASC-CM for 24 hours. Data are from a representative experiment repeated twice with similar results. **(B):** Quantitation of viable cells (green cells) under different culture conditions expressed as percentage compared with Con cells. **, $p < .01$ compared with CGN grown in Low K conditions. **(C):** Neutralization of IGF-1 attenuated the neuroprotective activity of ASC-CM. IGF-1-neutralizing ab (1 μl) or anti-human IgG was added to ASC-CM (1.15 μg of protein per microliter). The mixture was incubated at 37°C for 2 hours. Immediately after switching to Low K medium, 10 μl of ab-treated ASC-CM was added. Following an overnight incubation (approximately 16 hours), neuronal viability was quantified. *, $p < .05$ compared with Low K with 30% CM. Abbreviations: ab, antibody; CGN, cerebellar granular neurons; CM, conditioned medium; Con, control; Hi K, Basal Media Eagle without serum containing 25 mM K^+; IGF, insulin-like growth factor; Low K, Basal Media Eagle without serum containing 5 mM K^+.

vitro experiments using the well-validated model of primary developing rodent CGN, which undergo developmentally regulated apoptosis that peaks at the end of the first week of postnatal life [56]. Cultured CGN derived from rat or mouse cerebella at postnatal day 7 die of serum and potassium (K^+) deprivation via apoptosis unless they are provided with extrinsic survival factors [36, 57] or depolarizing concentrations of K^+ [24, 58]. Since neural apoptosis in this model is particularly blocked by the addition of IGF-1 [59], we assayed ASC-CM for the presence of IGF-1, first as part of a broader screen for growth factors using a antibody array blot (supporting information Fig. 1A) and then quantitatively by ELISA analysis of 1×- and 100×-concentrated ASC-CM, respectively. These analyses demonstrated that ASCs secrete detectable levels of IGF-1 (676 pg/ml) during growth in BME. To assess whether the levels in ASC-CM are sufficient to produce the observed biological effects, we determined the effect of neutralizing the activity of IGF-1 on the ability of ASC-CM to protect CGN cultured in conditions that promote apoptosis (Fig. 5). ASC-CM significantly protected CGN against serum and K^+ deprivation-induced neuronal death (Fig. 5B). A significant portion of this protective activity was attributed to IGF-1, as demonstrated by reduced neuroprotective activity of ASC-CM resulting from inactivation of IGF-1 by a neutralizing antibody (Fig. 5C).

ASC-CM Protection from Glutamate Excitotoxicity Can Be Blocked by a BDNF-Neutralizing Antibody

To further evaluate the neuroprotective activity of ASC-CM, we next examined the ability of ASC-CM to protect CGN against glutamate-induced excitotoxicity, a widely used model for research of stroke, Parkinson's disease, and Alzheimer's disease [43]. As shown in Figure 6A, ASC-CM significantly protected neurons against glutamate-induced excitotoxicity in a dose-dependent manner ($p < .001$ compared with untreated CGN). It has been demonstrated previously that the neurotrophic factor BDNF particularly blocks glutamate damage to neurons [37]; therefore, we assessed the presence of BDNF activity in ASC-CM and determined the contribution of this factor in protecting against glutamate-induced neurotoxicity. ASCs secrete detectable levels of BDNF (13.4 pg/ml) under the growth conditions used (supporting information Fig. 1B). Neutralization of BDNF nearly abolished the neuroprotective effect of ASC-CM in this model (Fig. 6B); thus, this is another important component contributing to the neuroprotective activity of ASC-CM.

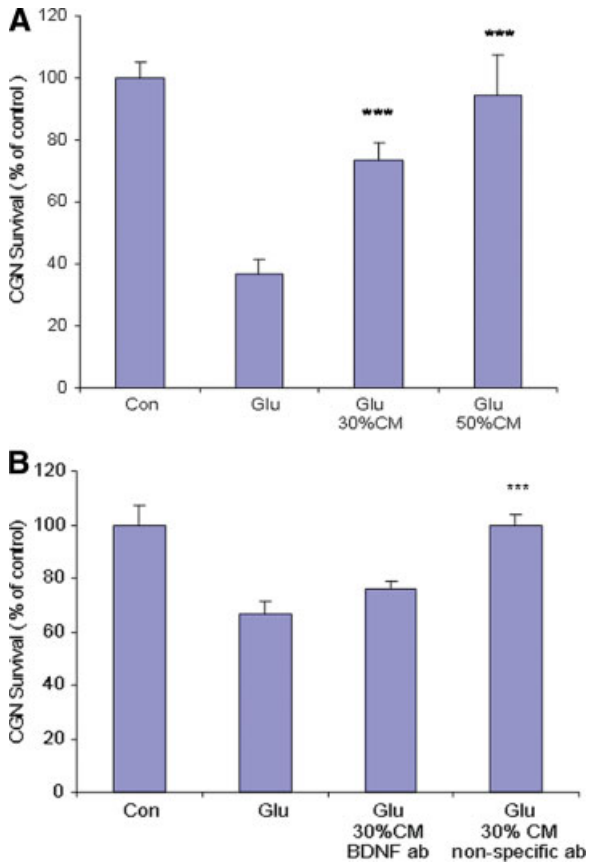

Figure 6. CM from cultured rat adipose-derived stem cells (ASC-CM) protection against glutamate excitotoxicity was blocked by a BDNF ab. **(A):** Rat CGN were cultured in Basal Media Eagle with 25 mM K^+ and 10% serum in the presence or absence of 30% or 50% (by volume) ASC-CM (30% or 50% CM, respectively). After 24 hours 50 μM glutamate was added to all wells except for those serving as the Con group, which also did not contain ASC-CM. The ability of ASC-CM to block glutamate toxicity was determined by assessing viability with fluorescein diacetate staining as described in Figure 6 and Methods and is expressed as a percentage of viable Con cells. **(B):** ASC-CM was pretreated with either BDNF ab or Con IgG (nonspecific ab) and subsequently added to the CGN cultures that were then challenged by 50 μM glutamate. ***, $p < .001$. Abbreviations: ab, antibody; BDNF ab, brain-derived neurotrophic factor-neutralizing antibody; CGN, cerebellar granular neurons; CM, conditioned medium; Con, control.

ASC-CM Protection from HI-Induced Injury Can Be Partially Blocked by Either IGF-1- or BDNF-Neutralizing Antibodies

To confirm the neuroprotective role of IGF-1 and BDNF in ASC-CM, neutralizing antibodies were again used to inactivate the individual activity of each protein before administering to HI-injured rats. As demonstrated by reduced protection from HI-induced lesion areas in the hippocampal and cortical regions of the brain after inactivation, the activities of both BDNF and IGF-1 significantly contribute to the neuroprotective activity of ASC-CM (Fig. 7).

DISCUSSION

The long-term goal of stem cell therapies has been to identify stem or progenitor cell types capable of replacing tissues lost to damage caused by disease or trauma. Although great strides

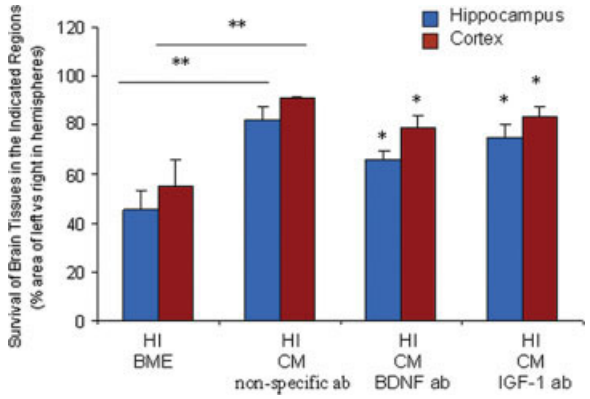

Figure 7. CM from cultured rat adipose-derived stem cells (ASC-CM)-mediated reduction of the lesioned area following HI injury was partially attenuated by neutralization of IGF-1 or the brain-derived neurotrophic factor activities. ASC-CM was pretreated with either IGF-1 ab ($n = 4$), BDNF ab ($n = 4$), or control IgG (nonspecific ab, $n = 4$) for 30 minutes before these mixtures or BME as a control ($n = 5$) was subsequently infused intravenously at 1 hour before the rats were subjected to HI injury. The area of tissue in the lesioned hippocampal or cortical hemispheres of each animal was analyzed as described in Figure 1. *, $p < .05$ compared with nonspecific ab only; **, $p < .01$ as compared with the BME. Abbreviations: ab, antibody; BDNF ab, brain-derived neurotrophic factor-neutralizing antibodies; BME, Basal Media Eagle; CM, conditioned medium; HI, hypoxic-ischemic; IGF-1 ab, insulin-like growth factor-1-neutralizing antibodies.

have been accomplished in determining the mechanisms governing de novo or forced differentiation of stem or progenitor cells for regenerative therapies in vitro, key technical hurdles of delivery remain to be solved to facilitate translation of cell-based therapies to the clinic. This is especially the case for neurological applications, particularly with respect to the brain, because of the baseline restriction in permeability by resulting from the BBB. As an alternative to invasive delivery methods required for overcoming the BBB, the convergent discoveries of resident neural progenitor cells in the adult brain, as well as molecules that stimulate homing (e.g., stroma-derived factor [SDF-1/CXCL12]) or differentiation (e.g., nerve growth factor) of these cells, has drawn much interest because of the potential for stimulating endogenous repair without the need for targeting donor cells to the brain [60–62]. Another potential approach builds on the current understanding that many beneficial effects observed in disease models with stem and progenitor cells result principally from trophic support provided in a paracrine manner from these cells [13, 31–34].

The mechanisms of action of secreted protein factors could be as diverse as stimulating receptor-mediated survival pathways, modulating inflammatory reactions, or promoting repair through inducing progenitor and stem cell homing and differentiation to replace lost tissues.

Our study focused on provision of the factors produced by ASCs, as a systemic bolus, and clearly demonstrates for the first time that ASC-CM effectively and potently blocks neuronal damage, tissue loss, and functional impairment in a model of neonatal HI injury-induced encephalopathy. The rat model used in this study is a well-validated surrogate of the human disorder, which suggests that ASC-CM may be an efficacious treatment to prevent or attenuate HI encephalopathy in human neonates [63, 64]. Indeed, in a parallel study we have found that ASC-CM derived from human ASCs manifests parallel neuroprotective activity in vitro (X.W., unpublished data). Importantly, it was demonstrated in this study that ASC-CM exerted potent neuroprotection when administered well after injury, thus supporting

the potential for therapeutic administration in humans, where treatment of human perinatal asphyxia is practical only well after the insult.

Penetration of brain tissues by factors in the conditioned medium is made possible in the context of hypoxia and ischemia as a result of a temporary disruption of the BBB between 24 and 72 hours [47–50], likely associated with the disruption of endothelial gap junctions, which occurs as a consequence of the local postischemic inflammatory response [51, 52]. Because HIE infants are intensively monitored following the injury, this time frame indeed presents a feasible opportunity to test whether administration of ASC-CM immediately following birth may limit brain damage as seen in the neonatal rats. It is also striking that the provision of ASC-CM yields long-term benefits in functional cognitive and memory behavior 2 months after HI, in the context that neonatal human HIE is associated with a high incidence of behavioral asymmetry and cognitive deficits that last in to adulthood [65, 66].

Whereas HI injury induced significant spatial learning and memory deficits at postnatal day 67 in neonates that did not receive treatment, there was very little abnormality in the behavior of rats treated with ASC-CM, either before or after HI, compared with sham animals, suggesting that sustained injury was nearly totally blocked by this treatment. Deficits in spatial (relational) memory are associated with damage to the hippocampal region [67, 68]. Animals in the control group had average losses of 48.8% of neurons in the hippocampus and 31.7% in the cortex. Administration of ASC-CM significantly attenuated tissue loss in this region, which correlated with enhanced performance in behavioral assessments. These observations suggest that ASCs produce activities that block neurodegenerative processes leading to cell death. One of the important factors governing delayed caspase-dependent cell death after neonatal HI is p38 [55]. This study has shown that HI-induced p38 phosphorylation was significantly inhibited by ASC-CM, suggesting one mechanism by which factors secreted by ASCs may be neuroprotective.

We have previously demonstrated that ASCs secrete bioactive levels of angiogenic and antiapoptotic growth factors. In this study, we found that ASCs secrete additional neurotrophic factors, including IGF-1 and BDNF, which have been shown in previous studies to potently protect neuronal cells from HI-induced brain damage [69, 70]. We have demonstrated that the accumulated levels of IGF-1 and BDNF are biologically relevant in assays of primary neurons subjected to apoptotic and excitotoxic injury stimuli, both processes that are known to be influenced by these proteins [24]. However, although neutralization of each of these factors attenuated the neuroprotective activity of ASC-CM in both in vitro and in vivo models, we cannot rule out the contribution of other factors for the HI model, such as, for instance, VEGF and HGF. Previous studies have examined the potential of BDNF and IGF in the model used here; however, an equivalent effect with the individual factors was observed only at much higher concentrations of each (5 μg of BDNF and 50 μg of IGF-1 delivered directly by intracerebroventricular injections [68, 69]), compared with the concentration present in ASC-CM (33.5 pg of BDNF and 1,690 pg of IGF-1 delivered systemically through i.v. injection). In addition, it is possible that in addition to neuroprotection, some factors in ASC-CM may stimulate migration and homing, as well as differentiation of brain-resident neuroprogenitors to replace lost tissues. Recent studies suggest that combinations of factors may activate synergistic signal transduction events in neuroprotective pathways [71]. We thus speculate that coadministration of synergistic neuroprotective agents, such as those present in ASC-CM, may provide benefit, particularly in the context of neurological disorders that engage multiple pathological pathways.

The elucidation of the factors in ASC-CM by proteomics analysis will identify the complement of key bioactive factors that contribute to neuroprotection in the HI model. Furthermore, systematic selective neutralization of individual and combinations of factors will allow definition of the critical combinations required to exert protection. The inclusion of additional models other than those used in this study, especially those directly related to HI injury, will provide additional mechanistic insights into the exact function of neurotrophic factors present in ASC-CM, as opposed to the simple demonstration in this that IGF-1 and BDNF factors released from ASCs are bioactive and actively protect neurons from injury-mediated cell death.

Conclusion

Our data suggest that ASC secrete bioactive factors including IGF-1 and BDNF that protect neurons in both in vitro and in vivo neuronal injury models.

Acknowledgments

This study was funded by a Charitable Contribution by the Hillary Dramas Foundation (to K.L.M.).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279–4295.
2 Zuk PA, Zhu M, Mizuno H et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001;7:211–228.
3 Safford KM, Hicok KC, Safford SD et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 2002;294:371–379.
4 Safford KM, Safford SD, Gimble JM et al. Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. Exp Neurol 2004;187:319–328.
5 Tholpady SS, Katz AJ, Ogle RC. Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. Anat Rec A Discov Mol Cell Evol Biol 2003;272:398–402.
6 Yang LY, Liu XM, Sun B et al. Adipose tissue-derived stromal cells express neuronal phenotypes. Chin Med J (Engl) 2004;117:425–429.
7 Ning H, Lin G, Lue TF et al. Neuron-like differentiation of adipose tissue-derived stromal cells and vascular smooth muscle cells. Differentiation 2006;74:510–518.
8 Kang SK, Putnam LA, Ylostalo J et al. Neurogenesis of Rhesus adipose stromal cells. J Cell Sci 2004;117:4289–4299.
9 Kang SK, Lee DH, Bae YC et al. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol 2003;183:355–366.
10 Ashjian PH, Elbarbary AS, Edmonds B et al. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg 2003;111:1922–1931.
11 Fujimura J, Ogawa R, Mizuno H et al. Neural differentiation of adipose-

derived stem cells isolated from GFP transgenic mice. Biochem Biophys Res Commun 2005;333:116–121.
12 Jin K, Greenberg DA. Tales of transdifferentiation. Exp Neurol 2003; 183:255–257.
13 Cai L, Johnstone BH, Cook TG et al. Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. STEM CELLS 2007;25:3234–3243.
14 Rehman J, Traktuev D, Li J et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004;109:1292–1298.
15 Nakagami H, Maeda K, Morishita R et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol 2005;25:2542–2547.
16 Sadat S, Gehmert S, Song YH et al. The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. Biochem Biophys Res Commun 2007;363:674–679.
17 Kumral A, Uysal N, Tugyan K et al. Erythropoietin improves long-term spatial memory deficits and brain injury following neonatal hypoxia-ischemia in rats. Behav Brain Res 2004;153:77–86.
18 Wei L, Han BH, Li Y et al. Cell death mechanism and protective effect of erythropoietin after focal ischemia in the whisker-barrel cortex of neonatal rats. J Pharmacol Exp Ther 2006;317:109–116.
19 Iwai M, Cao G, Yin W et al. Erythropoietin promotes neuronal replacement through revascularization and neurogenesis after neonatal hypoxia/ischemia in rats. Stroke 2007;38:2795–2803.
20 Escartin C, Boyer F, Bemelmans AP et al. Insulin growth factor-1 protects against excitotoxicity in the rat striatum. Neuroreport 2004;15: 2251–2254.
21 Kazanis I, Giannakopoulou M, Philippidis H et al. Alterations in IGF-I, BDNF and NT-3 levels following experimental brain trauma and the effect of IGF-I administration. Exp Neurol 2004;186:221–234.
22 Almli CR, Levy TJ, Han BH et al. BDNF protects against spatial memory deficits following neonatal hypoxia-ischemia. Exp Neurol 2000; 166:99–114.
23 Schäbitz WR, Sommer C, Zoder W et al. Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke 2000; 31:2212–2217.
24 D'Mello SR, Borodezt K, Soltoff SP. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: Possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 1997;17:1548–1560.
25 Wei X, Zhao L, Ma Z et al. Caffeic acid phenethyl ester prevents neonatal hypoxic-ischaemic brain injury. Brain 2004;127:2629–2635.
26 Carli G, Reiger I, Evans N. One-year neurodevelopmental outcome after moderate newborn hypoxic ischaemic encephalopathy. J Paediatr Child Health 2004;40:217–220.
27 Berger R, Garnier Y. Perinatal brain injury. J Perinat Med 2000;28: 261–285.
28 Vannucci RC. Hypoxic-ischemic encephalopathy. Am J Perinatol 2000; 17:113–120.
29 Pfenninger J, Bachmann D, Wagner BP. Survivors with bad outcome after hypoxic-ischaemic encephalopathy: Full-term neonates compare unfavourably with children. Swiss Med Wkly 2001;131:267–272.
30 Hou D, Youssef EA, Brinton TJ et al. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: Implications for current clinical trials. Circulation 2005; 112:I150–156.
31 Hardy SA, Maltman DJ, Przyborski SA. Mesenchymal stem cells as mediators of neural differentiation. Curr Stem Cell Res Ther 2008;3: 43–52.
32 Hess DC, Borlongan CV. Stem cells and neurological diseases. Cell Prolif 2008;41(suppl 1):94–114.
33 Capone C, Frigerio S, Fumagalli S et al. Neurosphere-derived cells exert a neuroprotective action by changing the ischemic microenvironment. PLoS ONE 2007;2:e373.
34 Meier C, Middelanis J, Wasielewski B et al. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. Pediatr Res 2006;59:244–249.
35 Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.
36 D'Mello SR, Galli C, Ciotti T et al. Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci U S A 1993;90:10989–10993.
37 Almeida RD, Manadas BJ, Melo CV et al. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 2005;12:1329–1343.
38 Rice JE III, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 1981;9: 131–141.
39 Han BH, Xu D, Choi J et al. Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. J Biol Chem 2002;277: 30128–30136.
40 Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 2nd ed. Sydney, Australia: Academic Press, 1986.
41 Uylings HB, van Eden CG, Hofman MA. Morphometry of size/volume variables and comparison of their bivariate relations in the nervous system under different conditions. J Neurosci Methods 1986; 18:19–37.
42 Khan IM, Miller DH, Strickland J et al. Brain kallikrein-kinin system abnormalities in spontaneously hypertensive rats. Hypertension 1995;25: 524–530.
43 Du Y, Bales KR, Dodel RC et al. Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. Proc Natl Acad Sci U S A 1997;94: 11657–11662.
44 Lin S, Yan C, Wei X et al. p38 MAP kinase mediates bilirubin-induced neuronal death of cultured rat cerebellar granule neurons. Neurosci Lett 2003;353:209–212.
45 Ni B, Wu X, Du Y et al. Cloning and expression of a rat brain interleukin-1beta-converting enzyme (ICE)-related protease (IRP) and its possible role in apoptosis of cultured cerebellar granule neurons. J Neurosci 1997;17:1561–1569.
46 Dodel RC, Du Y, Bales KR et al. Caspase-3-like proteases and 6-hydroxydopamine induced neuronal cell death. Brain Res Mol Brain Res 1999;64:141–148.
47 Ikeda T, Xia XY, Xia YX et al. Hyperthermic preconditioning prevents blood-brain barrier disruption produced by hypoxia-ischemia in newborn rat. Brain Res Dev Brain Res 1999;117:53–58.
48 Meng S, Qiao M, Lin L et al. Correspondence of AQP4 expression and hypoxic-ischaemic brain oedema monitored by magnetic resonance imaging in the immature and juvenile rat. Eur J Neurosci 2004;19:2261–2269.
49 Qiao M, Malisza KL, Del Bigio MR et al. Transient hypoxia-ischemia in rats: Changes in diffusion-sensitive MR imaging findings, extracellular space, and Na+-K+-adenosine triphosphatase and cytochrome oxidase activity. Radiology 2002;223:65–75.
50 Tuor UI, Kozlowski P, Del Bigio MR et al. Diffusion- and T2-weighted increases in magnetic resonance images of immature brain during hypoxia-ischemia: Transient reversal posthypoxia. Exp Neurol 1998;150: 321–328.
51 Cowell RM, Xu H, Galasso JM et al. Hypoxic-ischemic injury induces macrophage inflammatory protein-1alpha expression in immature rat brain. Stroke 2002;33:795–801.
52 Bona E, Andersson AL, Blomgren K et al. Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res 1999; 45:500–509.
53 Guilak F, Lott KE, Awad HA et al. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol 2006;206:229–237.
54 Hill IE, MacManus JP, Rasquinha I et al. DNA fragmentation indicative of apoptosis following unilateral cerebral hypoxia-ischemia in the neonatal rat. Brain Res 1995;676:398–403.
55 Hee Han B, Choi J, Holtzman DM. Evidence that p38 mitogen-activated protein kinase contributes to neonatal hypoxic-ischemic brain injury. Dev Neurosci 2002;24:405–410.
56 Wood KA, Dipasquale B, Youle RJ. In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. Neuron 1993;11:621–632.
57 Dudek H, Datta SR, Franke TF et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661–665.
58 Miller TM, Johnson EM Jr. Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. J Neurosci 1996;16:7487–7495.
59 Yamagishi S, Matsumoto T, Yokomaku D et al. Comparison of inhibitory effects of brain-derived neurotrophic factor and insulin-like growth factor on low potassium-induced apoptosis and activation of p38 MAPK and c-Jun in cultured cerebellar granule neurons. Brain Res Mol Brain Res 2003;119:184–191.
60 Imitola J, Raddassi K, Park KI et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci U S A 2004; 101:18117–18122.
61 Jin K, Galvan V. Endogenous neural stem cells in the adult brain. J Neuroimmune Pharmacol 2007;2:236–242.
62 Yamashima T, Tonchev AB, Yukie M. Adult hippocampal neurogenesis in rodents and primates: Endogenous, enhanced, and engrafted. Rev Neurosci 2007;18:67–82.
63 Vannucci RC, Connor JR, Mauger DT et al. Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res 1999;55:158–163.

64 Vannucci RC, Vannucci SJ. Perinatal hypoxic-ischemic brain damage: Evolution of an animal model. Dev Neurosci 2005;27:81–86.
65 Balduini W, De Angelis V, Mazzoni E et al. Long-lasting behavioral alterations following a hypoxic/ischemic brain injury in neonatal rats. Brain Res 2000;859:318–325.
66 Ikeda T, Mishima K, Yoshikawa T et al. Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. Behav Brain Res 2001;118:17–25.
67 Altemus KL, Almli CR. Neonatal hippocampal damage in rats: Long-term spatial memory deficits and associations with magnitude of hippocampal damage. Hippocampus 1997;7:403–415.
68 Henriette van Praag CFD, Ira B. Black dissociation of motor hyperactivity and spatial memory deficits by selective hippocampal lesions in the neonatal rat. J Cog Neurosci 1994;6:321–331.
69 Brywe KG, Mallard C, Gustavsson M et al. IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? Eur J Neurosci 2005;21:1489–1502.
70 Cheng Y, Gidday JM, Yan Q et al. Marked age-dependent neuroprotection by brain-derived neurotrophic factor against neonatal hypoxic-ischemic brain injury. Ann Neurol 1997;41:521–529.
71 Digicaylioglu M, Garden G, Timberlake S et al. Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. Proc Natl Acad Sci U S A 2004;101:9855–9860.

See www.StemCells.com for supporting information available online.