Timing of Cord Blood Treatment After Experimental Stroke Determines Therapeutic Efficacy

Jennifer D. Newcomb,*† Craig T. Ajmo, Jr.,‡ Cyndy D. Sanberg,§ Paul R. Sanberg,*†‡§ Keith R. Pennypacker,‡ and Alison E. Willing*†‡

*Center of Excellence for Aging and Brain Repair, University of South Florida College of Medicine, Tampa, FL 33612, USA
†Department of Neurosurgery, University of South Florida College of Medicine, Tampa, FL 33612, USA
‡Department of Pharmacology and Molecular Therapeutics, University of South Florida College of Medicine, Tampa, FL 33612, USA
§Saneron CCEL Therapeutics, Inc., Tampa, FL 33612, USA

Embolic stroke is thought to cause irreparable damage in the brain immediately adjacent to the region of reduced blood perfusion. Therefore, much of the current research focuses on treatments such as anti-inflammatory, neuroprotective, and cell replacement strategies to minimize behavioral and physiological consequences. In the present study, intravenous delivery of human umbilical cord blood cells (HUCBC) 48 h after a middle cerebral artery occlusion (MCAo) in a rat resulted in both behavioral and physiological recovery. Nissl and TUNEL staining demonstrated that many of the neurons in the core were rescued, indicating that while both necrotic and apoptotic cell death occur in ischemia, it is clear that apoptosis plays a larger role than first anticipated. Further, immunohistochemical and histochemical analysis showed a diminished and/or lack of granulocyte and monocyte infiltration and astrocytic and microglial activation in the parenchyma in animals treated with HUCBC 48 h poststroke. Successful treatment at this time point should offer encouragement to clinicians that a therapy with a broader window of efficacy may soon be available to treat stroke.

Key words: Human umbilical cord blood; Middle cerebral artery occlusion (MCAo); Inflammation; Infarct core; Therapeutic window

INTRODUCTION

The American Stroke Association has reported that every 45 s someone in the US suffers from stroke and every 3 min someone dies from one (26). It is estimated that tax payers paid $53.6 billion for this disease in 2004 alone. Currently, the thrombolytic, recombinant tissue plasminogen activator (rtPA) is the only FDA-approved treatment that can offer a 33% success rate for recovery from ischemic stroke (21). However, rtPA has considerable risks (intracranial hemorrhage and recurrent stroke) and limitations (must be used within 3 h of stroke onset and only with embolic stroke) (21,26). Only 3–5% of stroke victims arrive at the hospital within this therapeutic window and of those only 29% are eligible for rtPA. In actuality, less than 1% of the 700,000 stroke victims in the US each year have a chance at a full recovery with clinical intervention. The remaining victims can only expect supportive and rehabilitative care.

The primary reason there are so few treatment options for an ischemic event in the brain is that the “core” area of the infarct adjacent to the vessel in which blood flow is blocked or reduced is thought to lose cells through excitotoxicity-induced necrosis. Hence, the vast majority of stroke research is focused on only arresting the spread of neuronal death in the brain through treatment of cells in the peri-infarct area, or “penumbra,” the region in which cells die more slowly through apoptosis. Many of these experimental treatments have aimed only at reducing inflammation or enhancing neuroprotection, and none have been proven effective (14,40).

Two events cause neurodegeneration. There is a direct effect of glutamate excitotoxicity due to the sudden drop in oxygen and nutrients that begins immediately upon loss of blood flow (34). An indirect inflammatory reaction to the neurodegeneration, such as generation of free oxygen radicals and nitric oxide (6,16,22), activation of microglia within the parenchyma (15,35), and invasion of other inflammatory cells by way of chemotaxis and leakage across the compromised blood–brain barrier (BBB) (7,9,10,45) also occurs. This process begins later but endures longer. A successful treatment
must have a multifaceted anti-inflammatory and neuroprotective ability to respond to all of these insults. Human umbilical cord blood cells (HUCBC) have been used in experimental models of injury and disease over the past several years and show encouraging leaps toward development of such an all-inclusive treatment. Intravenous transplantation of the mononuclear fraction of HUCBC has successfully ameliorated deficits caused by spinal cord injury (31), myocardial infarction (11), traumatic brain injury (19), and neurodegenerative diseases and injury in rats (8,32,38,41,42).

Initially, cord blood was thought to contain an ideal source of cells to be used in cell replacement therapy for neurological pathology because of its high proportion of hematopoietic stem cells, which can be induced to express neural proteins (3,30). It has since been discovered in the stroke model, that once transplanted, these cells do not promote functional and neurological recovery via replacement, but instead modulate the immune response to ischemia as well as providing direct neuroprotection (39). Because the majority of delayed damage incurred by a stroke results from the ensuing inflammatory response, HUCBC’s mechanisms of action are of particular interest. Further, for HUCBC treatment to be optimally effective, the timing of its administration must coincide with the time course of injury-induced inflammatory mechanisms.

A recent in vitro migration study revealed that a significantly greater number of HUCBC migrated to homogenized extracts of striatal and hippocampal tissue at 48 and 72 h after middle cerebral artery occlusion (MCAo) injury in rats (25). Newman et al. (25) also found that monocyte chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) were elevated 48 h poststroke, suggesting their participation in the migration of the HUCBC. The present study was designed to determine if rats subjected to MCAo and treated with HUCBC within this 48–72-h window fared better than at other treatment time points. A HUCBC dose of 10^6 cells was chosen based on Vendrame et al.’s (38) study establishing that this number of cells was the threshold dose upon which physiological and behavioral recovery could be measured. Though 10^6 HUCBC had provided the animals with the greatest benefit when given 24 h post-MCAo, the threshold dose was used in the current study to more sensitively measure recovery based on the treatment’s temporal administration. We intravenously administered HUCBC at various time points following MCAo in rats and analyzed motor behavior 2 and 4 weeks posttreatment. In addition, histological and immunohistochemical staining was performed to measure infarct volume and presence of various inflammatory cells.

**MATERIALS AND METHODS**

**Animals**

Sixty-four adult male Sprague-Dawley rats weighing 200–250 g were used. They were housed in pairs in a temperature- and humidity-controlled room under a 12-h light–dark cycle. Food and water were available ad libitum. The animals were randomly assigned to one of six transplantation time points following MCAo [3 h (n = 9), 24 h (n = 9), 48 h (n = 9), 72 h (n = 9), 7 days (n = 10), and 1 month (n = 9)] with MCAo-only animals (n = 9) serving as a control. Once it was determined that the optimal treatment time point was 48 h poststroke, a subgroup of 42 rats (200–250 g) was matched to one of three groups based on their average percentage drop in cerebral blood pressure upon arterial occlusion (Table 1): sham surgery (n = 6), MCAo only (n = 22), or MCAo + HUCBC (treatment 48 h post-MCAo, n = 14). Sham rats were sacrificed 2 days post-surgery, MCAo-only rats were sacrificed at 2 (n = 9), 4 (n = 6), and 7 days (n = 7) post-MCAo and MCAo + HUCBC-treated rats were sacrificed at 4 (n = 8) and 7 days (n = 6) post-MCAo (2 and 5 days posttreatment). All experimental procedures were performed in accordance with the NIH guidelines for the care and use of animals. Veterinary expertise was available when needed.

**MCAo Surgery**

The animals were anesthetized with isoflurane (2–5% in O2; at 2 L/min). The right common carotid, external carotid, internal carotid, and pterygopalantine arteries were isolated using blunt dissection. The external carotid was ligated, cut, and an intraluminal suture (40 mm length of 4.0 monofilament) was inserted through it, into the internal carotid and up to the origin of the middle cerebral artery (approximately 25 mm). Once in place the suture was tied in permanently and the incision closed. All rats received preoperative injections of Ketophen® (10 mg/kg, IM), atropine (0.25 mg/kg, SC), and Baytril® (20 mg/kg, IM). Baytril® and Ketophen® were continued for 3 days post-MCAo to prevent infection and control pain, respectively.

**Table 1.** Average Percent Change in Cerebral Blood Pressure Upon Middle Cerebral Artery Occlusion

<table>
<thead>
<tr>
<th>Group Membership</th>
<th>Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>−2.23 (1.74)</td>
</tr>
<tr>
<td>MCAo only 2 days</td>
<td>−84.80 (1.01)</td>
</tr>
<tr>
<td>MCAo only 4 days</td>
<td>−84.93 (1.64)</td>
</tr>
<tr>
<td>MCAo only 7 days</td>
<td>−82.86 (1.71)</td>
</tr>
<tr>
<td>MCAo + CB 4 days</td>
<td>−84.78 (1.22)</td>
</tr>
<tr>
<td>MCAo + CB 7 days</td>
<td>−85.13 (1.13)</td>
</tr>
</tbody>
</table>
Doppler Monitoring

The rats were anesthetized and maintained with isoflurane (2–5% in O₂ at 2 L/min) and a small hole was drilled through the skull at 1 mm posterior and 4 mm right lateral to bregma. A fiber optic filament was fed through the hole to rest on dura, taking care not to disturb the cortex, and was connected to a Laser Doppler (Moor Instruments, Devon, England) that recorded cerebral blood pressure changes throughout the surgery. The data were saved to a computer for later analysis. Criteria for inclusion in the study was ≥70% drop from baseline blood pressure.

Cell Preparation and Transplantation

The HUCBC (Cambrex Corp., East Rutherford, NJ & Sanerion CCEL Therapeutics, Tampa, FL) were thawed rapidly at 37°C, washed in HBSS plus HEPES, and centrifuged (1000 rpm for 10 min) three times. Viability was determined using the trypan blue dye exclusion method. Cell concentration was adjusted to 10⁶ in 500 μl. The animals were anesthetized with 5% isoflurane in O₂ at 2 L/min, the penile vein exposed, and a 28-gauge needle inserted into the lumen of the vein for cell delivery. HUCBC obtained from different donors were evenly distributed among the treatment groups. All animals in the first study were given intraperitoneal injections of the immunosuppressant cyclosporine A (10 mg/kg) at the time of transplantation and repeated each day until sacrifice. Rats in the Doppler-monitored subgroup did not receive cyclosporine A based on the report by Pan et al. (28).

Behavioral Testing

Rats in the first study were behaviorally tested prior to stroke, providing a baseline to which behavior at 2 and 4 weeks posttransplant was compared.

Step Test. The step test is a sensitive measure of motor asymmetry. Rats were held at a 75° angle with one forepaw placed on a table. They were dragged 1 m at constant speed in the direction of their placed paw and the number of steps taken was recorded. Both the right and left paws were tested in random order.

Accelerated Rotorod Test. The rotorod (Omnitech Rotoscan, Columbus, OH) consisted of a Plexiglas box (66 x 51 x 9 cm) within which a 70-mm-diameter activity wheel was suspended. Rats were tested for motor coordination on the revolving wheel that gradually accelerated from 0 to 40 rpm over a 3-min period. The test was performed three times a day for 3 consecutive days. Latency to fall and speed at fall were recorded.

Tissue Preparation

The rats were transcardially perfused with phosphate buffer (PB), 7.4 pH, to clear out all blood followed by 4% formaldehyde in 0.1 M PB to fix the tissues. The brains and organs (heart, lungs, liver, kidneys, spleen, thymus, and bone marrow) were removed, postfixed for 24 h, and then cryopreserved in 20% sucrose prior to cutting 30-μm frozen sections on a cryostat (Mikron Instruments, San Marcos, CA).

Histology and Immunohistochemistry

Thionin (Sigma-Aldrich, St. Louis, MO). Brain sections were chosen 1 mm apart beginning at −1.7 mm anterior to bregma and continuing through 3.3 mm posterior to bregma to encompass the infarct area most affected by blockage of the MCA. The slide-mounted sections were hydrated, stained for Nissl substances with thionin for 90 s, washed in HBSS plus HEPES, and centrifuged (1000 rpm for 10 min) three times. Viability was determined using the trypan blue dye exclusion method. Cell concentration was adjusted to 10⁶ in 500 μl. The animals were anesthetized with 5% isoflurane in O₂ at 2 L/min, the penile vein exposed, and a 28-gauge needle inserted into the lumen of the vein for cell delivery. HUCBC obtained from different donors were evenly distributed among the treatment groups. All animals in the first study were given intraperitoneal injections of the immunosuppressant cyclosporine A (10 mg/kg) at the time of transplantation and repeated each day until sacrifice. Rats in the Doppler-monitored subgroup did not receive cyclosporine A based on the report by Pan et al. (28).

α-Naphthyl Acetate Esterase (Monocytes, Sigma-Aldrich). Solutions were prepared per kit instructions. Tissue slides were incubated in prewarmed α-naphthyl solution (40°C) and protected from light. The slides were then rinsed in distilled water and counterstained with hematoxylin solution for 2 min, rinsed with tap water, and allowed to air dry prior to coverslipping with glycerol.

Glial Fibrillary Acidic Protein (GFAP, Dako, Carpenteria, CA). Tissue slides were incubated in polyclonal primary antibody (1:750) overnight at 4°C. Slides were then rinsed and incubated in rhodamine-conjugated secondary antibody (Molecular Probes, Eugene, OR, 1: 200) for 2 h at room temperature. Slides were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA).

MHC II (Ox-6, Serotec, Raleigh, NC). Tissue slides were incubated in the monoclonal antibody (1: 300) overnight at 4°C. Slides were then rinsed and incu-
bated for 2 h in FITC-conjugated secondary antibody (Molecular Probes, 1:200) at room temperature. Slides were coverslipped with Vectashield.

In Situ Apoptosis Detection (TUNEL). Frozen sections were prepared and treated per kit instructions (NeuroTACS, R & D Systems, Minneapolis, MN). Briefly, thaw-mounted cryostat sections were incubated in Neuropore for 30 min, rinsed in two changes of DNase-free water for 2 min each, and then immersed in quenching solution for 5 min. Slides were washed with PBS for 1 min, immersed in 1× TdT labeling buffer for 5 min, and then labeling reaction mix was pipetted onto each section and incubated for 60 min after which the slides were immersed in stop buffer for 5 min. The sections were then washed twice with PBS for 2 min each and then streptavidin HRP solution was pipetted onto each section and incubated for 10 min and again washed in two changes of PBS for 2 min each. The slides were then immersed in DAB solution for 5 min, washed in two changes of distilled water for 2 min each, and then immersed in blue counterstain for 60 s. The sections were then rinsed in tap water, ammonium water, dehydrated, and coverslipped with Permount. Positive and negative controls were prepared on each slide.

Statistical Analysis

All data were analyzed using analysis of variance (ANOVA). Post hoc analysis was performed using Newman-Keuls or Fisher’s Least Significant Difference (protected t-test) if the overall ANOVA was significant. If homogeneity of variance tests were significant, the Mann-Whitney U-test was used. Simple linear regression was used to determine the correlation between behavior and intact brain.

RESULTS

Motor Behavior Improves

Figure 1 shows the behavioral recovery that the animals made after receiving HUCBC 48 h poststroke. Animals that received a transplant 48 h following MCAo also showed significantly greater motor improvement in the accelerated rotord test 4 weeks posttransplant than MCAo-only controls (W = 2.88, p < 0.05), 3-h (W = 7.89, p < 0.01), 24-h (W = 5.81, p < 0.01), and 72-h (W = 4.41, p < 0.01) transplant groups (Fig. 1A). Animals receiving transplants 48 h after MCAo took significantly more steps with their left paw (contralateral to lesion) than all other groups in the step test: MCAo only (U = 0, p < 0.01), 3 h (U = 1.5, p < 0.01), 24 h (U = 5.5, p < 0.01), 72 h (U = 3, p < 0.01), 7 days (U = 6, p < 0.01), and 1 month (U = 3, p < 0.01) (Fig. 1B). In many cases the rats achieved a full recovery based on both of these behavioral tests.

Neuronal Rescue

Sections taken between the anterior commissure and subthalamic nucleus for all treatment time point groups showed a substantial neuroprotective effect of HUCBC treatment when given 48 h after stroke compared to other treatment time points and MCAo-only controls (F = 2.810, p = 0.024) (Fig. 2). Here it was clear that HUCBC treatment at 48 h prevented degradation of the cytoarchitecture. Brain tissue remained intact in many of the animals at this treatment time. Additionally, behavior and infarct volume in this model were significantly correlated (r² = 0.174, p < 0.01; regression line, y = 0.32X + 47.42) (Fig. 3).

Inflammatory Response Is Stunted

Immunohistochemical and histochemical analysis was performed to assess the degree of the local inflammatory response across the groups. As expected, there was diminished astrocytic and activated microglial staining in animals with smaller infarcts. Specifically, there was very little GFAP and Ox6 (astrocytes and activated microglia, respectively) staining in animals that were treated with HUCBC 48 h post-MCAo compared to MCAo-only rats (Fig. 4A–D). Similarly, peripheral immune cell invasion (granulocytes and monocytes) was also decreased (Fig. 4E–H).

Infarct Evolution in a Rat Over 7 Days

Once it was determined that treatment at 48 h contributed to the greatest physiological and behavioral recovery, a subgroup of animals was subjected to MCAo while monitored with Laser Doppler to verify that the MCAo technique was consistent in its ability to produce a severe drop in cerebral blood flow (≥70% drop in pressure) (33). Animals in this subgroup reproduced our findings and extended the description of cell death and inflammation after MCAo and HUCBC transplantation. TUNEL-positive cells indicative of apoptotic death were observed in the right ventral lateral striatum (core) over the course of 7 days following MCAo (Fig. 5). HUCBC treatment at 48 h may inhibit proapoptotic pathways, possibly through expression of antiapoptotic genes such as Bcl-2 or Bcl-xL. A noticeable infarct was not found in many of the animals in this group (Fig. 6). There was a significant difference in infarct size between the groups (F = 5.33, p = 0.0011). Specifically, rats sacrificed 4 days post-MCAo had significantly greater infarct size than rats sacrificed 2 days post-MCAo (t = 2.19, p < 0.05), HUCBC-treated rats sacrificed 4 days post-MCAo (t = 2.56, p < 0.05), HUCBC-treated rats sacrificed 7 days post-MCAo (t = 3.67, p < 0.01), and sham-operated rats (t = 4.07, p < 0.01). Similarly, rats sacrificed 7 days post-MCAo had significantly greater infarct size than HUCBC-treated rats sacrificed at the same time (t =
Figure 1. HUCBC transplanted 48 h post-MCAo induces optimal behavioral recovery. Rats subjected to MCAo and treated with HUCBC 48 h later demonstrate near normal motor behavior: (A) staying on a revolving rod for longer periods of time and at a higher RPM in the accelerated rotorod test and (B) displaying very little asymmetry in gait on the step test compared to rats treated at other time points and MCAo-only controls. *p < 0.05; **p < 0.01.

3.10, p < 0.01) and sham-operated rats (t = 3.51, p < 0.01). Of particular interest is the lack of significant difference in infarct size between rats sacrificed 2 days post-MCAo and all HUCBC-treated and sham-operated rats. These data show that a much larger percentage of cells can still be rescued at later time points than expected based on earlier studies. Moreover, immunohistochemistry of astrocytes and activated microglia showed that both inflammatory cells increased in number up to 4 days post-MCAo, diminishing by 7 days (Fig. 7). Positive staining was reduced in HUCBC-treated animals.

DISCUSSION

The beneficial effects of HUCBC treatment following stroke have been well established. There are obvious reductions in infarct volume when these cells are given 24 h poststroke and that reduction has been found to be dose dependent (38). Though the standard 10⁶ cell dose can lessen some behavioral stroke symptoms, Vendrame et al. (38) found that 10⁷ cells were necessary to significantly reduce the size of the lesion. In the current study, however, 10⁶ HUCBC were adequate to rescue not only the cells in the penumbra but also to limit the size of the core when intravenously delivered 48 h poststroke.

This dose was also able to restore motor impairments to baseline measures. Rats subjected to unilateral permanent MCAo display hallmark motor asymmetry in behavioral performance tests and intravenous treatment of HUCBC administered 24 h poststroke has been shown in previous studies to mitigate these symptoms (5,38, 41,42). To this point, treatment at 24 h with the threshold dose of 10⁶ cells had been set based on positive, though somewhat variable, behavioral outcomes (38,41, 42). Transplanting at 24 h post-MCAo requires 10⁷ cells to reliably maximize recovery. Therefore, it may not be surprising that 10⁶ cells at 24 h did not produce better recovery in this study. HUCBC treatment is more effective at 48 h than at 24 h to the extent that it takes one tenth the number of cells to induce significant behavioral recovery. This low dose at this treatment time point
Figure 2. HUCBC provides neuroprotection when given 48 h post-MCAo. Thionin staining was performed on predetermined sections throughout the striatal and hippocampal tissue harvested 1 month posttransplant. Photomicrographs depict the extent of infarct in representative animals from each treatment group and controls. (A) MCAo only, (B) 3 h, (C) 24 h, (D) 48 h, (E) 72 h, (F) 7 days, (G) 1 month. The bar graph (H) shows the percent volume of the ipsilateral (stroked) hemisphere compared to the contralateral (nonstroked) hemisphere for each group.

Figure 3. Behavioral and physiological recovery are significantly correlated. This is the first report to show a relationship between improvement in motor behavior and extent of infarction. When animals were treated with HUCBC 48 h subsequent to MCAo a significant positive relationship was found between the number of steps made on the rat’s contralateral (affected) side and the measure of intact brain tissue.
Figure 4. Photomicrographs demonstrating a neuroprotective effect of HUCBC treatment at 48 h. Following MCAo, GFAP and OX-6 immunohistochemistry staining showed a dramatic astrocytic (A) and microglial (C) response, respectively, 1 month posttransplant. IV treatment with mononuclear HUCBC at 48 h considerably reduces this astrocytic (B) and microglial (D) activation. Similarly, histological staining with α-naphthyl acetate esterase and naphthol AS-D chloroacetate esterase identified inflammatory infiltrates, such as monocytes and granulocytes, respectively, were also reduced in 48-h HUCBC-treated animals (E and G, respectively) compared to MCAo-only controls (F and H, respectively). Granulocytes found in 48-h treated rats were mostly confined to vessels (H). All scale bars: 100 µm.

Figure 5. Apoptotic cell death in the core is reversed. TUNEL-positive cells in the core reach their peak 2 days post-MCAo (B); however, many were still seen at 4 (C) and 7 days (D) after ischemia. When HUCBC treatment is given at 48 h there are no cells found undergoing apoptosis 4 and 7 days post-MCAo (E and F). Sham-operated rats served as a control (A). Scale bar: 100 µm.
Figure 6. Infarct expansion continues beyond 2 days post-MCAo. Thionin staining clearly shows that the infarct is small 48 h after stroke (A), expanded significantly by 4 days (B), but takes at least 7 days to complete (C). A noticeable infarct was not found in many of the animals in the HUCBC-treated rats sacrificed both 4 and 7 days post-MCAo (D and E, respectively). (F) Sham-operated rats. The graph represents the size comparison of the intact tissue within the ipsilateral (stroked) hemisphere in relation to the contralateral (“normal”) hemisphere for each group of rats (G). These results challenge the strongly held beliefs that the core neurons cannot be rescued and also that the infarct is well established by 2 days post-MCAo.

Figure 7. Reduced astrocytic and microglial activation. Following MCAo, GFAP and OX-6 immunohistochemistry staining showed a dramatic astrocytic (A$_1$–C$_1$, scale bar: 50 µm) and microglial (A$_2$–C$_2$, scale bar: 200 µm) response. IV treatment with mononuclear HUCBC 48 h post-MCAo considerably reduced this astrocytic (D$_1$ and E$_1$, scale bar: 50 µm) and microglial (D$_2$ and E$_2$, scale bar: 200 µm) activation. (F$_1$ and F$_2$) Sham operated.
ameliorated both behavioral consequences of MCAo and concomitantly reduced infarct volume, a significant correlation of which had yet to be attained prior to this study.

Clearly, these data demonstrate that ischemic injury is more receptive to HUCBC treatment 48 h poststroke than at other time points. Further, the timing of treatment is more important than the dose of cells because the effective dose at 48 h is an order of magnitude smaller than the optimal dose at earlier time points. These results are even more noteworthy in light of the observation that neurons within the ischemic core are spared or rescued this late after stroke; the actual core size is much smaller than expected, indicating that many more cells than previously believed are still viable even 48 h after injury. The cells within the infarct core cannot be dying predominately by necrosis, the globally accepted mechanism of death for cells in that area. Instead, delayed death through apoptotic mechanisms is the more likely cause of cell death. In fact, it has been observed that cells in the core show features similar to that seen with apoptosis and not necrosis (27,43). If apoptosis is the rule and not the exception in the core then it may be possible to rescue these cells because the end stages of death may not occur for 3–4 days after ischemia/hypoxia (17), thereby opening up the therapeutic window.

The inflammatory cascade following ischemia is a complicated interplay of molecular and biochemical events in which a delicate balance of excitatory and inhibitory signals determines cellular fate. Immediately following a stroke, a cascade of neuronal degeneration, regeneration, and repair begins. Microglia secrete cytotoxic substances such as glutamate, nitric oxide, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which have been shown to damage cells after stroke (45); the microglial response peaks at 2–3 days poststroke (20,45). Similarly, the BBB is disrupted as early as 3 h after stroke, allowing an influx of peripheral immune cells. Parallelising these events, astrocytic activation begins at 3 h; between 24 and 96 h astrocytic S-100β, which stimulates nitric oxide production, increases (7). Further, GFAP expression increases within 24 h and remains elevated for at least 8 days; reactive astrocytes also express TNF-α and IL-1β, both of which peak prior to 24 h (13,18) and activate the transcription factor nuclear factor-kB (NF-kB) and subsequently a number of genes that respond to brain injury (29). NF-kB has a ubiquitous role in the inflammatory response to injury. In neurons, NF-kB can promote survival (29), while in microglia and astrocytes, this transcription factor increases expression of genes associated with inflammation (24). It is during this peak inflammatory response that HUCBC seem to be of the most benefit.

HUCBC cells may be recruited to the infarct site within the brain by one of two mechanisms. The first is that of passive diffusion across a damaged BBB. However, disruption of the BBB begins within the first 3 h after MCAo (12). While there is a second peak between 24 and 72 h in the transient occlusion model that may be associated with reperfusion injury, this is not the case with the permanent MCAo model (1). In contrast, in the permanent MCAo, after an initial period of vasogenic edema, there is a more gradual increase in BBB breakdown that peaks approximately 6 days post MCAo and is accompanied by cellular edema (23), yet the optimal timing of HUCBC delivery is 48 h. The second mechanism is that of chemokines promoting recruitment of specific cell types to the site of injury. Some of these inflammatory proteins—MCP-1, CINC-1, and intercellular adhesion molecule-1 (ICAM-1)—reach peak expression 48 h after stroke (4,37,44). Therefore, it is not likely a coincidence, considering the timing of these events, that we see the greatest therapeutic benefit from HUCBC transplanted 48 h poststroke. It is possible that the heightened chemotactic expression is needed to recruit HUCBC to the site of injury in order for them to block the inflammatory response and provide protection to compromised neurons. Treatment directed against inflammatory processes that is delivered too early or too late does not help recovery and may even worsen outcome in some cases. However, in the early hours following ischemia, microglial and astrocytic response may be neuroprotective (2). HUCBC given too early may interfere with the body’s early natural attempt to defend against injury, leading instead to exacerbation of the damage.

The immune system and its response to injury are highly dynamic, making the clinical efficacy of a single intervention limited. The therapeutic potential of HUCBC appears to be multifaceted in its ability to enhance neuronal survival through modulation of the immune response to an ischemic event and providing trophic support to starved cells in the ischemic zone. It is only through the ability of these cells to provide both anti-inflammatory and neuroprotective effects that HUCBC can reduce the size of the infarct core (39), rescuing neurons from apoptotic cell death and thereby improving motor function. Further, the CD34+ subset of HUCBC has also been shown to induce revascularization, supporting neurogenesis (36). Moreover, the current study clearly demonstrates that the therapeutic window for treating stroke can be extended out to at least 48 h, when treatment efficacy is maximized. When temporal administration of HUCBC is optimized, the number of cells that must be delivered is an order of magnitude smaller than at nonoptimal transplant times. Such a protracted window of effective therapeutic intervention is encouraging. This or similar treatments could potentially reach...
the majority of stroke sufferers instead of the small percentage of patients that fit the current therapeutic criteria. Used in conjunction with other stroke therapies that treat early consequences of stroke could be an even more powerful therapeutic regimen. What’s more, HUCBC treatment, or a pharmacological mimic, could also be extended to other ailments in which an inflammatory element of a disease or injury creates more harm than healing.

ACKNOWLEDGMENTS: The authors would like to thank Melanie Thomas, Fabio Ferrari, and Lisa Collier for their technical assistance and dedication to this project. This study was supported in part by grants to A.E.W. from Florida Biomedical Research Program (BM039) and the American Heart Association (#0355183B). P.R.S. is cofounder of Saneron CCEL Therapeutics. C.D.S. is an employee of and A.E.W. is a consultant to Saneron CCEL Therapeutics. Both A.E.W. and P.R.S. are inventors on cord blood-related patent applications.

REFERENCES

10. Gasche, Y.; Copin, J. C.; Chan, P. K. Type IV collagenases and blood-brain barrier breakdown in brain ischemia.

27. Onténte, B.; Couriard, C.; Braudeau, J.; Benchoua, A.;


