Cord Blood Cell Therapy for Ischemic Stroke

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Cord Blood Cell Therapy for Ischemic Stroke

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Infusion of the “mononuclear fraction” of human cord blood cells (HUCBC), which is composed of immature blood cells and hematopoietic progenitors, is known to reduce neurobehavioral deficits in rats subject to middle cerebral artery occlusion (MCAO). When MCAO rats are infused with $10^6$ cells 24 hours after the induction of the stroke, their motor function improves. To extend these findings, we first examined the behavioral recovery of MCAO rats in the presence of increasing doses of HUCBC. The recovery in behavioral performance seen with measurements of spontaneous activity and motor deficits, depended on the amount of cells delivered, with $10^6$ HUCBC being the threshold for significant behavioral recovery. Measurements of the ischemic volume revealed an inverse relationship between HUCBC dose and damage volume, which reached significance at the higher HUCBC doses ($10^7$ and $3-5x10^7$ cells). Moreover, investigation of the distribution of the intravenously injected cells showed that HUCBC were localized to the injured brain hemisphere and the spleen. Given these findings, we hypothesized that there may be a role of HUCBC in the modulation of the peripheral or brain-localized immune response that is normally evoked after stroke.

Results on the effect of HUCBC infusion on splenocytes indicated that HUCBC treatment prevented the alterations in splenocyte type (CD8+ depletion) and function (T-cell suppression) induced by stroke. In particular, examination of cytokine production
from splenocyte cultures of HUCBC-treated MCAO rats revealed increased production of IL-10 and decreased production of IFN gamma relative to MCAO rats. Microglia (immunostained with a CD11b antibody) and B cells (identified with the B220 cell marker) that were increased after MCAO were dramatically decreased after HUCBC treatment. Proinflammatory cytokines such as TNF-alpha, IL-1beta and IL-2 were upregulated after MCAO surgery and their expression was abrogated after HUCBC infusion. All these findings indicate that the action of HUCBC may be specifically related to the modulation of the stroke-induced inflammatory response, providing a possible mechanism by which cord blood cells have been repeatedly reported to promote functional recovery from ischemic injury.
CHAPTER ONE

INTRODUCTION

1.1 Epidemiology of stroke

Although the worldwide incidence of stroke is unknown, in the United States it is well documented that stroke affects approximately 700,000 Americans per year (HDSS 2004 Update). Of these, only one-third achieves full recovery, one-third succumbs within the following 12 months, and the remaining third are affected by permanent disabilities (HDSS 2004 Update). Although, stroke affects all age groups, the risk increases dramatically with aging [more than 90% of all strokes occurring in people of age 55 and older] (HDSS 2004 Update). The risk factors associated with stroke include: hypertension, tobacco use, diabetes, high alcohol intake, poor diet, excessive weight, family history of cardiovascular disease, and previous episodes of stroke or transient ischemic attacks (TIAs) (Green GP 2004). Stroke is already the third leading cause of death and the leading cause of disability, but as the American population median age is expected to increase over the next two decades, stroke looms an ever-increasing threat for the American society.
1.2 Pathology of Stroke

The term “stroke” generally refers to any cardiovascular accident that occurs in the central nervous system (CNS). This group of vascular pathologies of the brain includes hemorrhagic accidents (due to the rupture of a blood vessel) as well as disorders that follow the occlusion of a blood vessel, i.e. ischemic injuries. These latter “ischemic strokes” encompass the majority of strokes (more than 80%) (HDSS 2004 Update).

About 50 percent of ischemic strokes are caused by cerebral thrombosis, which is caused by the formation of a blood clot within cerebral arteries that are damaged by arteriosclerosis. Thrombosis can affect large-vessels such as the carotid, middle cerebral, or basilar arteries as well as small-vessels (lenticulostriate, basilar penetrating, medullary) causing a type of thrombotic stroke known as a lacunar stroke.

The other 50 percent of ischemic strokes is caused by cerebral embolism, a type of ischemic stroke that occurs as a result of blockage of circulation to a portion of the brain parenchyma by an embolus. The embolus can be composed of a variety of substances (such as fat or air) but more frequently is derived from a blood clot, mainly originating from the heart or the walls of the carotid arteries. The embolus moves freely through the blood stream until it lodges within an artery too small to allow its passage, blocking the blood behind it from passing through.

Within seconds to minutes of the loss of perfusion to an area of brain parenchyma, an ischemic cascade is triggered, the result of which is irreversible damage or infarction of the area lacking blood flow (core), around which there is an area of potentially reversible injury, called the “penumbra”. At the cellular level, the electrophysiological properties of the neurons subject to ischemia are altered, as a consequence of the sudden depletion of
ATP in response to hypoxia, and the consequent failure of cell membrane ion-transport systems (Diarra, Sheldon et al. 1999; Sheldon and Church 2004). The resulting intracellular flow of calcium and other cations causes the release of several neurotransmitters, such as large amounts of glutamate, which activates N-methyl-D-aspartate (NMDA) and other excitatory receptors (Liu, Grossman et al. 2001). These cells then become depolarized, causing additional calcium influx, additional release of glutamate, and local amplification of the initial ischemic insult. This enormous calcium influx leads to neuron cell death by activating various degradative enzymes, including calpain and metalloproteases which degrade laminin and other components of the brain matrix (Fukuda, Fini et al. 2004).

A number of toxic compounds are generated by this process, including free oxygen radicals and nitric oxide, which themselves induce additional neuronal damage (Liu, Grossman et al. 2001). Within hours to days after a stroke, an inflammatory reaction is evoked which provokes the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) within the ischemic brain (Buttini, Appel et al. 1996). Although initially protective, this inflammatory reaction if chronically present extends the damage to the surrounding tissue (del Zoppo, Becker et al. 2001). Without therapeutic intervention, the penumbra will be consumed by this cascade of multiple insults and inevitably lose its functionality completely.
1.3 The goal of stroke therapy

The central goal of stroke therapy is to salvage the penumbral region. This can be obtained by 1) reducing the duration of ischemia, i.e. re-instating the blood flow to the compromised area; or 2) limiting the severity of cell injury as seen with strategies based on neuronal protection. The ischemic cascade offers multiple steps at which such strategies can be applied and many interventions aimed at stopping this cascade of events are being investigated. A key factor is the timing of restoring cerebral blood flow, given that clinical studies have concluded that reperfusion must occur within 3 hours in order to salvage the penumbra (NINDS 1995).

1.3.1 Thrombolytic Therapy: Tissue-Plasminogen Activator (t-PA)

Thrombolytics work by breaking up the clot responsible for obstructing the blood flow, allowing blood to re-flow through the occluded vessel. In 1995, the tissue-Plasmonogen Activator (t-PA) Study conducted by National Institute of Neurological Disorders and Stroke (Department of Health and Human Services at the National Institute of Health, Bethesda, MA) published the results of two randomized clinical trials on 600 stroke patients, which concluded that there was an improvement by thrombolytic t-PA use in patients when treated within 90 minutes of stroke onset (NINDS 1995). The U.S. Food and Drug Administration (FDA) approved t-PA as a treatment for stroke in 1996, with its employment being restricted to a 3 hours time window from stroke onset. This treatment remains the only effective therapeutic option for stroke. A recent study published in the Lancet (Hacke, Donnan et al. 2004) shows the results of an extensive analysis of about 3,000 stroke patients, who were randomized for treatment with t-PA or a placebo, in six
controlled clinical trials: the findings demonstrate that t-PA treatment within 3 hours of stroke onset induced significant recovery. Moreover, the study suggests that t-PA given up to 4 hours after the stroke onset may be of benefit, but the authors point out that there is a decreasing effectiveness with almost no benefit at all when t-PA treatment is done at 6 hours post-stroke onset. While there is strong evidence that early treatment with t-PA can improve a stroke patient's possibility of complete recovery, only an estimated 2 to 5 percent of all eligible stroke patients receive thrombolytics treatment.

1.3.2 Neuroprotective Strategies

Neuroprotective therapies are treatments intended to block the cellular, biochemical, and metabolic processes of neuronal death during or following the ischemic insult. They include an array of pharmacologic interventions, such as antagonists of glutamate receptors, calcium channel blockers, anti-edema agents and neurotrophic factors. Studies on stroke animal models have shown that these substances have a beneficial effect as seen by either behavioral measures and/or in reduction of infarct volume of the stroke brain. For instance, N-methyl-D-aspartate (NMDA) receptor antagonists, (such as Conantokin-G and the compound SM-18400) are known to reduce infarct volume in stroked rats (Ohtani, Tanaka et al. 2000; Williams, Ling et al. 2002). Calcium channels blockers such as nimodipine (Korenkov, Pahnke et al. 2000), have also been examined in rodent models of cerebral ischemia and shown to possess neuroprotective properties. Some evidence also suggests that magnesium sulfate (MgSO\textsubscript{4}) may inhibit ischemic damage by blocking calcium channels. Intravenous delivery of 5% MgSO\textsubscript{4} (90 mg/kg)
up to 6 hours post-stroke is proven to improve neurological outcome and reduce brain infarct volume in rats (Yang, Li et al. 2000).

More recently, neurotrophic factors have also been introduced into the experimental setting with very promising results. In stroked rats, intravenous infusion of brain-derived neurotrophic factor (BDNF) (starting at 30 minutes after MCAO surgery and prolonged for 2 hours) induced a reduction in infarct volume visible at 5 hours post-stroke (Berger, Schabitz et al. 2004). When applied in synergy with hypothermia, the same treatment can dramatically inhibit the release of glutamate in the corpus striatum (Berger, Schabitz et al. 2004). Recently, viral vector mediated gene transfer of neurotrophic factors has been considered as a new therapeutic alternative for ischemic brain injury.

Intraventricular gene delivery of glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), BDNF, insulin-like growth factor-1 (IGF-1) or vascular endothelial growth factor (VEGF) up to 6 hours after the ischemic insult blocked neuronal death induced by occlusion of the bilateral carotid arteries in gerbils (Shirakura, Inoue et al. 2004).

1.3.3 Anti-inflammatory Strategies

The idea of blocking the inflammatory reaction induced by ischemia in the brain has been the basis of several experimental therapeutic approaches for stroke. Anti-inflammatory agents were first introduced in this arena for their anti-coagulant properties. Agents like aspirin, ibuprofen and serine protease inhibitors, such as the urinary trypsin inhibitor (UTI), have been shown to ameliorate behavioral deficits in rodent models of ischemic injury and concomitantly reduce infarct volume (Cole, Patel et al. 1993; Yano, Anraku et
Great success in the experimental setting has been obtained with the employment of blockers of the infiltration of inflammatory cells to the brain parenchyma. For example, intravenous application of interferon-beta (IFN-beta), a cytokine with anti-inflammatory properties, attenuated the development of brain infarction by 70% in stroked rats (Veldhuis, Derksen et al. 2003). This effect was obtained when IFN-beta infusion was begun 6 hours after onset of stroke symptoms. Immunohistochemistry at 24 hours after stroke onset showed that IFN-beta almost completely blocked the migration of inflammatory cells such as neutrophils and monocytes into the CNS parenchyma. Other studies have applied alternative molecular strategies to prevent the infiltration of inflammatory cells, such as pharmacologically blocking adhesion molecules. For example, the dyad CD18-Inter Cellular Adhesion Molecule 1 (ICAM-1) attracted a lot of attention when antibodies blocking their interaction induced neurological improvement and reduced infarct size in stroked rodents (Zhang, Chopp et al. 1994; Hartl, Schurer et al. 1996). The potential application of these strategies has been confirmed by several experimental studies, but clinical trials based on such strategies have yielded less enthusiasm. In 1995 the Enlimomab Acute Stroke trial concluded that intravenous administration of the murine anti-human ICAM-1 antibody did not induce any benefit in stroke patients (DeGraba 1998). Despite several logistic and pragmatic reasons that could explain such failure, therapeutic approaches based on similar strategies still remain confined to the experimental setting.
1.3.4 Clinical trials on Stroke

As of 2004, there are 38 stroke trials on-going worldwide (AHA 2004). Some of these are focused on intra-arterial revascularization strategies, based on the idea that it is efficacious to restore the blood flow either before the vessel has been fully obstructed or after the stroke. One of these trials is the Carotid and Vertebral Artery Transluminal Angioplasty Study (CAVATAS) that determines the benefits and risks of percutaneous transluminal angioplasty of the carotid and vertebral arteries in patients with symptomatic and asymptomatic cerebrovascular disease. Other trials focus on the control of physiological parameters that are normally altered after an ischemic insult. The Controlling Hypertension and Hypotension Immediately Post stroke (CHHIPS) Trial involves almost 2,000 patients across the United Kingdom and aims at the controlling blood pressure with depressor therapy within 12 hours post-stroke. Other on-going trials apply neuroprotective strategies such as intravenous delivery of magnesium sulfate, after the demonstration that magnesium sulfate is safe and well-tolerated in stroke patients. The Intravenous Magnesium Efficacy in Stroke Trial (IMAGES) trial is coordinated by the University of California Los Angeles and is designed to test the efficacy of MgS$_2$OH$_3$ given at 12 hours of stroke onset. Finally, there are on-going stroke trials that test the efficacy of vitamins treatment in patients with recurrent Transient ischemic Attacks (TIA). The Vitamins to prevent Stroke (VITATOPS) trial administers vitamin supplements of B12, B6 and folate to patients who had a TIA within the last 7 months.
1.4 The rodent middle cerebral artery occlusion model of stroke.

Several animal models of stroke have been developed in order to test new therapeutic options for treatment of sequelae of brain ischemia. Among all these models, the middle cerebral artery occlusion (MCAO) that we use in our laboratory is the most commonly employed. Other stroke models of cerebral ischemia include bilateral carotid occlusion (Eklof and Siesjo 1972), intracranial compression that produces ischemia and increased intracranial pressure (Ljunggren, Ratcheson et al. 1974), unilateral carotid occlusion plus hypoxia (Nakajima, Ishida et al. 1999), compression of the neck with a cuff (Siemkowicz and Hansen 1978), common carotid artery or internal carotid artery occlusion with thrombotic blood clot (Overgaard, Sereghy et al. 1992) as well as four vessel occlusion (Pulsinelli and Brierley 1979).

All these stroke models do not provide a model of “focal” ischemia, i.e. an ischemic lesion limited to specific brain areas, as the MCAO model does and as it occurs in the neuropathology of clinical stroke. Therefore, the occlusion of the MCA remains the model of choice that most closely mimicks the “real” scenario of brain ischemic events. Additionally, the MCA is also the vessel most commonly occluded in embolic strokes (Symon, Dorsch et al. 1975).

The MCAO model involves the selective occlusion of the right or left MCA. During the last three decades it has been subjected to several variations in order to optimize technical aspects, outcome reproducibility and reliability (Tamura, Graham et al. 1981; Belayev, Alonso et al. 1996; Zhang, Chopp et al. 1997).

Most of these studies are done on rodents such as mice and rats although early studies employed felines and primates because these could better mimic the clinical neurological
syndromes following stroke in men. However, issues related to handling, costs and animal-ethical concerns induced a shift to rodent models (Garcia, Lossinsky et al. 1978). The first MCA occlusion procedures, from the 70’s, involved the ligation and severance of the MCA, after frontotemporal craniotomy (Robinson, Shoemaker et al. 1975; Robinson 1979). This procedure was able to induce a lesion limited to the rat cerebral cortex with a minimal variation in the lesion size between animals. However, the lesion area did not appear to consistently resemble the ischemic area damaged in human MCA stroke (Robinson 1979). Tamura et al. (Tamura, Graham et al. 1981) and later Longa et al (Longa, Weinstein et al. 1989) described a technique in which a suture was inserted intraluminally from the bifurcation of the common carotid artery. Since the length of the suture corresponds to the distance from its insertion to the base of the MCA (about 20mm, depending on the size of the animal), the tip of the suture would reach the base of the MCA and occlude it theoretically. A schematic drawing of this procedure is illustrated in Figure 1.1. This procedure could be performed without craniotomy with a simple neck incision and blunt dissection of the common carotid artery. Moreover, on subsequent pathological examination the cerebral lesion resembled more closely that seen in human MCA stroke (Tamura, Graham et al. 1981). Furthermore, this procedure was reversible, allowing the suture to be removed at a later time (Longa, Weinstein et al. 1989).

This option has indeed allowed other investigators to modify this procedure and perform a “transient” MCAO: removal of the suture after a certain time allows a reperfusion of the ischemic area, mimicking what can happen in an embolic or thrombo-embolic occlusion, when spontaneous removal of the embolus allows blood to re-flow through the
Figure 1.1
Schema of inferior view of rat brain with its underlying vasculature. ACA, MCA, PCA: anterior, middle and posterior cerebral arteries; ECA and CCA: external and common carotid arteries. The embolus is inserted through the ECA to the origin of the MCA.
ischemic area. This variation of performing a transient occlusion has been adopted by several investigators (Li, Chopp et al. 2000; Chen, Sanberg et al. 2001).

Other variations of this MCAO procedure are for the most part technical, mainly involving different points of insertion of the suture and innovations in surgical dissection techniques. In the procedure described by Belayev et al. (Belayev, Alonso et al. 1996) the suture is inserted through the proximal external carotid artery, then into the internal carotid artery and finally into the base of the MCA. This procedure includes the isolation and coagulation of the branches of the external carotid artery and ligation of the pterygopalatine artery. This technique has been used successfully in several reports (Willing, Jiang et al. 2002; Belayev, Khoutorova et al. 2003) and it is the technique currently used in our laboratory. Specifically, we employed the «permanent» occlusion model, i.e. without removal of the suture after placement (Willing, Lixian et al. 2003; Willing, Vendrame et al. 2003).

1.5 Hematotherapy for repair of ischemic brain

1.5.1 Cell therapy for stroke

Cell transplantation has recently emerged as a therapeutic alternative for neurodegenerative diseases including stroke. This field has received increasing attention after the revolutionary discovery that the brain also possesses a regenerative capacity, through the presence of neural stem cells present even in the adult human brain (Reynolds and Weiss 1992; Song, Stevens et al. 2002). Neural stem cells which are able to self-renew and commit to neuronal, astrocytic or oligodendroglial lineages are
primarily derived from human embryos (Reubinoff, Itsykson et al. 2001). However, the idea of harvesting these cells from such sources has generated ethical concerns.

Two alternative cell sources are represented by the human embryonic teratocarcinoma cells lines, which can be differentiated in vitro into post-mitotic neurons, or by stem cells isolated from hematopoietic tissues (such as adult bone marrow and cord blood), which demonstrate the capacity to attain neuronal and glial properties in vitro, but not derived from embryonic sources.

1.5.2 Human embryonic teratocarcinoma cells lines

The human embryonal carcinoma cell line (NTera-2/D1 or NT2 cells) are capable of differentiating into postmitotic neuron-like cells (hNT or NT2N cells) after exposure to retinoic acid (Pleasure and Lee 1993). Studies of NT2N cells transplanted into the CNS of non-diseased rodents showed that NT2N cells survived, integrated into the cerebral microenvironment, and developed molecular and structural characteristic of neurons (Trojanowski, Mantione et al. 1993). These cells can engraft and survive up to one year in rodent brains, without reverting to a tumorigenic state (Trojanowski, Mantione et al. 1993). Additionally, NT2N cells have been transplanted into the striatum of ischemic rats, showing grafting and survival at 6 months post-transplant, in addition to significant amelioration of functional stroke-induced deficits (Borlongan, Tajima et al. 1998). Based on these animal studies, a stroke phase I clinical trial employing NT2N cells was started in 2000 at the University of Pittsburgh (PA) (Kondziolka, Wechsler et al. 2000). This trial included 12 patients (aged 44 to 75 years) with basal ganglia infarct and fixed motor deficits, with a stroke clinically stable for at least 2 months (that occurred 6 months to 6
years previously. Imaging evaluations showed no adverse cell-related effects of the allograft (being these cells derived from a human cell line and transplanted into unrelated human recipients). Neurological scores showed improvement in six of the twelve patients and positron emission tomography scans at 6 months confirmed the presence of viable cells (Kondziolka, Wechsler et al. 2000). Autopsy findings in the brain of a phase I clinical stroke trial patient implanted with human NT2N cells (who died of causes unrelated to the stroke), found the transplanted cells adjacent to the infarct area at 27 months after surgery, with no evidence of development of neoplasm (Nelson, Kondziolka et al. 2002). These findings indicated that implanted NT2N cells could survive for a time greater than 2 years in the human brain without adverse effects, supporting the idea that therapies based on implantation of human embryonal carcinoma cell lines can be feasible and non-deleterious.

1.5.3 Bone marrow cells

Hematopoietic stem cells (HSC) derived from bone marrow were first identified for their ability to reconstitute blood lineages, and they were subsequently shown to differentiate into other cell types including neuroectodermic cells, such as neurons and glial cells. Despite some skepticism regarding the capacity of these cells to generate cells of such dissimilar lineage, an ever-increasing body of evidence suggests that development of neurons and astrocytes from bone-marrow-derived HSC’s is a bona-fide phenomenon. In addition to hematopoietic stem cells, bone marrow contains non-hematopoietic precursors cells, which are also referred to as mesenchymal cells or bone marrow stromal stem cells (BMSC), which have also been shown to differentiate into glia and neurons in vitro.
Several independent reports have confirmed the observation that systemically infused BMSCs can repopulate a number of non-hematopoietic tissues (Pereira, O'Hara et al. 1998). To assess whether BMSCs could also reconstitute CNS tissues, bone marrow cells genetically marked with a retroviral tag were injected intravenously in sublethally irradiated WBB6F1yJ-KitW/KitW-v mice (considered as good recipients for bone marrow transplant because they possess genetically defective hematopoietic system) (Eglitis and Mezey 1997). Weeks after the injection, tagged cells were observed in the brain where they were widely distributed in the cortex, hippocampus, thalamus, cerebellum and brain stem. These cells expressed either microglial markers (F4/80) or GFAP, suggesting that precursors resident in the bone marrow were able to reconstitute CNS glial populations (Eglitis and Mezey 1997). Others have confirmed this observation (Kopen, Prockop et al. 1999; Nakano, Migita et al. 2001) and further demonstrated that infused bone marrow derived cells can also develop neural traits after grafting into the brain, as seen with the expression of NeuN (Brazelton, Rossi et al. 2000; Mezey, Chandross et al. 2000), NSE (Mezey, Chandross et al. 2000), class III beta-tubulin and the 200kD neurofilament (Brazelton, Rossi et al. 2000).

BMSCs have also been transplanted intracerebrally. When injected into the striatum of albino rat brain, BMSC cells engrafted and migrated from the transplant site to different CNS areas along known pathways of neural stem cell migration (Azizi, Stokes et al. 1998), indicating that these cells can respond to local cues. Moreover, after the engraftment these cells lost markers typical of cultured BMSCs, and developed phenotypes similar to astrocytes.
In light of these results in non-diseased animals, bone marrow derived cells have been subsequently studied in animal models of neurodegenerative disease, with the idea that these cells might also be capable of reconstituting damaged tissues and consequently restoring brain function. In rodent models of stroke, transplantation of BMSCs either intrastriatally or intravascularly found that the BMSCs homed to the ischemic boundary zone and bore neuronal and glial antigens (Li, Chopp et al. 2000; Chen, Li et al. 2001; Li, Chen et al. 2001). The same investigators have reported that BMSCs can induce the in situ expression of vascular endothelial growth factor (VEGF) and its receptor thereby promoting neo-vascularization processes in the penumbral region (Chen, Zhang et al. 2003).

Not all the investigators believe that a real neuronal/glial differentiation of these cells is possible. Even accepting the idea that BM cells truly develop a neuronal/glial trait in vivo, the functionality of these cells as neurons or glia has not yet been proven. One emerging idea is that bone marrow derived cells may induce the differentiation of resident totipotent stem cells into functional cells that reconstitute damaged neurons. An increase in endogenous neural stem cell proliferation within the sub-ventricular zone and the hippocampus has been observed after BMSCs transplantation (Mahmood, Lu et al. 2001). Alternatively, the observed functional recovery may be a result of the increased expression of neurotrophic/growth factors. This phenomenon could be a function of either the endogenous production of growth factors induced by BMSCs or by delivery of these factors from the transplanted cells (Chen, Katakowski et al. 2002; Mahmood, Lu et al. 2004). In vitro studies employing TBI-conditioned BMSCs cultures have shown a time-dependent increase in BDNF, NGF, VEGF, and hepatocyte growth factor (HGF),
indicating a responsive production of these growth factors by the BMSCs (Chen, Katakowski et al. 2002). Recent reports suggest additional mechanisms that transplanted BMSCs may undergo. For instance, the expression of transplanted stem cells by neural phenotype may be explained by their fusion with endogenous cells (Terada, Hamazaki et al. 2002). This hypothesis, known as the “fusion theory”, is supported by in vitro co-culture experiments showing embryonic stem cells are able to fuse with bone marrow cells or neural stem cells (Terada, Hamazaki et al. 2002; Ying, Nichols et al. 2002). Cell fusion may indeed have a beneficial function, for instance transplanted cells may provide lacking genetic material to a cell otherwise undergoing necrosis. However, the issue that has yet to be resolved is whether expression of a diploid nuclear material may on the contrary lead to tumorogenetic processes.

1.5.4 Cord Blood Cells for Brain Repair

Accumulating evidence supports the idea that similar to bone marrow cells, cord blood cells undergo a phenotypic conversion into neuronal and glial lineages when exposed to defined culture conditions (Sanchez-Ramos, Song et al. 2001; Bicknese, Goodwin et al. 2002). Human umbilical cord blood cells (HUCBC) treated with retinoic acid (RA) and nerve growth factor (NGF) expressed molecular markers such as Musashi-1 (a specific marker for neural stem cells) and beta-tubulin III (structural proteins characteristic of neurons) (Sanchez-Ramos, Song et al. 2001). Also treatment of HUCBC with fibroblast growth factor (FGF) and epidermal growth factor (EGF) induces the expression of neural and glial markers as seen by Western immunoblots and immunocytochemical detection
of both beta-tubulin III and glial fibrillary acidic protein (GFAP) (Bicknese, Goodwin et al. 2002).

These findings led to the idea that cord blood cells could mimic the beneficial effects seen after bone marrow cell transplantation in animal models of neurodegenerative diseases. Therefore, cord blood cells have been used in models of stroke and traumatic brain injury, subsequently demonstrating that cord blood could also rescue motor deficits induced in these models. The most advanced work with cord blood as a treatment for neurodegeneration has been conducted on stroke animal models. The first report of benefit from HUCBC transplantation in an animal model of stroke occurred in 2001, when HUCBC was infused in the MCAO model and significant functional recovery was observed (Chen, Sanberg et al. 2001). Treatment at 24 hours after MCAO with $3 \times 10^6$ HUCBC injected into tail vein was reported to improve functional recovery, as evidenced by the rotarod test and Modified Neurological Severity Score (mNSS) (Chen, Sanberg et al. 2001). In the same study, treatment at 7 days after MCAO with the same amount of cells significantly improved function only on the mNSS. Another study concluded that the behavioral recovery induced in MCAO rats by $10^6$ HUCBC when delivered intravenously 24 hours post-stroke was comparable if not better than the recovery observed after intra-cerebral transplantation of 250,000 cells (Willing, Lixian et al. 2003). Investigators showed a similar recovery between the two groups in spontaneous activity and the passive avoidance task, while in the step test, significant improvements were found only after intravenous delivery of the HUCBC.
1.6 Current Clinical Use of Cord Blood Cell Therapy

Cord blood has been used extensively as an alternative source of hematopoietic stem cells in allogeneic stem-cell transplantation for the treatment of acquired and genetic diseases. The first related human umbilical cord blood (HUCB) transplant was performed in the seventies in a child with acute lymphoblastic leukemia (Ende and Ende 1972) and since then, thousands of HUCB transplants have been performed worldwide, with the majority performed as therapy for haematological malignancies (Lewis 2002). From these early experiences some clear advantages of using HUCB cells have become evident.

First, the HUCB cells are readily available. Contrary to bone marrow cells, whose harvest requires a bone marrow puncture, cord blood cells can be obtained from the umbilical cord of any healthy neonate. Importantly, HUCBC can be easily cryopreserved and stored in cord blood cell banks until needed for the transplant. These cryopreserved cord blood cells have been reported to show no difference in engraftment capabilities when banked up to 3 years (Goodwin, Grunzinger et al. 2003).

Secondly, there is a lower incidence of graft versus host disease (GVHD) with HUCB transplants compared to bone marrow transplants. This has allowed the usage of cord blood cell transplants from un-related donors with a minimal incidence of immune reactions. This advantage is probably related to the immunologic immaturity that cord blood cells have been shown to possess (Lewis 2002).

Thirdly, the risk of viral transmission with HUCB transplant is minimal. Bone marrow transplant can be jeopardized by the transmission of pathogens from the donor to the host, that can fatally infect the recipient. One of those is the Epstein-Barr virus (EBV), which is responsible for the onset of lymphoproliferative disorders. While bone marrow
transplant are generally accompanied by a significant incidence of EBV transmission, after cord blood transplants the incidence of EBV-associated lymphoproliferative disorders is low (Barker, Martin et al. 2001).

1.7 What is cord blood?

Cord Blood is the blood contained in the placenta and umbilical cord that is normally discarded after delivery of the neonate. Cord blood can be collected after clamping and cutting the umbilical cord, immediately after the birth of the baby. The actual part of the cord blood that is used for cell transplant is the “mononuclear fraction”, a fraction of the blood containing mainly mononuclear cells that is obtained by cell gradient separation techniques. Cord blood banks collect the umbilical cord blood, isolate the mononuclear fraction and cryopreserve it. Cryopreservation of these cells allows for long-term storage, permitting their use for future transplants. The mononuclear fraction is a heterogeneous population of cells that are distinguished from each other by the expression of different cell surface antigens. The stem cell subset is represented by cells bearing the CD34 molecule, a common marker for human hematopoietic stem cells. Literature reports that this fraction constitutes less than 1 percent of the entire mononuclear fraction (Pranke, Failace et al. 2001). This means, for instance, that in an HUCBC transplant of 100,000 cells, approximately 1000 to 2000 cells will be hematopoietic stem cells. Some investigators have identified some subclasses of cells within the CD34 stem cell fraction. One subtype of CD34 positive cells bear the c-KIT transmembrane tyrosine kinase growth factor receptor (CD117), whose ligand is the stem
cell factor (SCF) (Laver, Abboud et al. 1995). The CD117-SCF interaction is thought to be responsible for the adhesion of stem cells to the microenvironment (Laver, Abboud et al. 1995). Another subtype of the CD34 positive cells is represented by cells bearing CD34 and CD133. These cells have been shown to express the VEGF and other angiogenic factors, suggesting a role of these cells in the regulation of hematopoiesis and the generation of new capillaries at the injury site (Pomyje, Zivny et al. 2003). A third subset of CD34 cells is one that does not bear the CD38 marker. This fraction, CD34 positive but CD38 negative, is considered a more immature fraction and has been correlated strictly with the regenerative capacity of stem cells (Encabo, Mateu et al. 2003). In addition to the CD34 positive stem cell population, lymphocytes, monocytes and a small fraction of cells bearing a heterogeneous array of surface markers make up the remaining cells within the mononuclear fraction (Pranke, Failace et al. 2001). The lymphocytes are reported to constitute from 30 up to 45 percent of the entire fraction (Pranke, Failace et al. 2001; Nagamura-Inoue, Mori et al. 2004). This variability is probably due to different methodologies of separation of the mononuclear fraction. Alternatively, it may attributable to the variability between different babies’ blood and the immune status of the mother. About half of the lymphocytes are reported to be T cells, and the other half B cells. The T cells are approximately 80% CD4 positive with the remaining 20% being CD8 positive T cells. Expansion of specific T cell subsets can be done in vitro, with the use of specific cytokines such as interleukin-7 or interleukin-2 or the SCF (Sanchez, Alfani et al. 2003). The fraction of monocytes is reported in the literature as being quite variable, somewhere between 10 to 30 percent of the whole mononuclear fraction (Pranke, Failace et al. 2001; Sorg, Andres et al. 2001). These cells
express antigens characteristic of monocytes, such as CD14, CD19, CD11b and CD26L (Sorg, Andres et al. 2001). The remaining cells of the mononuclear fraction consists of various cell types; including: dendritic cells which do not express any specific marker, but can be morphologically distinguished from other mesenchymal derived cells as well as a CD31 positive population (also called PECAM-1) which is generally expressed by mature hematopoietic cells (Pranke, Failace et al. 2001).

Figure 1.2 illustrates a typical flow cytometry analysis of the composition of cells in the mononuclear fraction of cord blood, as typically employed in our laboratory. The stem cell subset (CD34+ cells) accounted for less than 1% of the entire fraction. Cells that are CD11b positive (such as monocytes) represented about 36% of the fraction, and CD4 positive T cells are almost 50%. B cells (as stained for the B cell marker B220) occupy a fraction of about 16%.

1.8 Conclusion

The work enclosed in this dissertation is broke down in three main chapters. First, we determined whether there was relationship between number of cord blood cells infused and behavioral/histological outcomes (Chapter Three). Secondly, we focused on the investigation of the possible effect of cord blood intravenous injection on the peripheral immune system, particularly in the spleen (Chapter Four). Finally, we explored the role of cord blood cells on the regulation of the inflammatory events that follow ischemia in the brain (Chapter Five).
Figure 1.2
Flow cytometry analysis on the cord blood mononuclear fraction. Composition of the cord blood mononuclear fraction as determined through flow cytometry.
CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 The Middle Cerebral Artery Occlusion Rat Model

2.1.1 Animal housing and maintenance

Adult Sprague Dawley rats (average body weight range: 175.0 ± 25.0 g) were used in all these experiments. Animals were housed in the Vivarium facility of the College of Medicine of the University of South Florida. They were allowed to acclimate for 7 days before undergoing any experimental procedure and kept under 12 hours dark/light conditions in a temperature-controlled room with water and chow *ad libitum*. Vivarium personnel replenished water and chow daily and routinely performed cage cleaning.

2.1.2 Surgical procedure

All surgical procedures were performed in surgical rooms of the Vivarium facility of the College of Medicine (University of South Florida), according to the guidelines of the National Institute of Health as approved by the University of South Florida Institutional Animal Care and Use Committee. The rats were anesthetized with Isofluorane mixed with 2 to 5 percent of oxygen. Induction of the anesthesia was performed delivering the gas mixture at the flow rate of 5 liters per minute. When the animal was in deep sleep (as determined by nonresponsiveness to toe pinch) the anesthesia was decreased to the flow...
of 2 liters per minute and maintained as such for the entire surgical procedure. The neck area was shaved and covered with Betadine solution (Abbott). With the use of blunt surgical techniques, cutaneous and subcutaneous tissues and the submandibular salivary gland were dissected free to allow the exposure of the right common carotid artery. This artery was carefully dissected free of the tenth cranial nerve. The internal and external carotid arteries were exposed and their small collateral vessels cauterized. The external carotid was then ligated proximally and distally and subsequently severed with a sharp instrument. The pterygopalatine artery was then isolated and tied off with a 5-0 suture.

The embolus used to induce the vessel occlusion was a commercially available nylon line of the diameter of 4 um that was cut to a length of 40 mm. The embolus was inserted through the external carotid artery into the internal carotid to the origin of the middle cerebral artery (approximately 25 mm from point of entry). The diagram in Figure 1.1 (Chapter 1) shows a pictorial representation. Afterwards, the embolus was tied in permanently and all the tissues re-positioned in their correct anatomical location. The neck incision was closed with 3-0 suture. Once all technical procedures were concluded, the anesthesia was stopped, the animal was placed in a clean cage on a warming pad and observed until awakening.

2.1.3 Post-surgical pharmacological and physical support

Immediately after the surgical procedure, all the rats received an injection with Ketofen (0.4 ml intramuscularly) and Baytril (0.1 ml subcutaneously) as a control of post-surgical pain and infectious reactions. These injections were repeated for four days after the
surgical procedure. All animals were under the continuous observation of the Vivarium staff.

2.2 Cord Blood Cells Transplantation

2.2.1 Preparation of Cryopreserved Cells

Cord blood cells were donated from a cord blood cell bank (Saneron CCEL Therapeutics, Inc., Tampa FL). The mononuclear fraction was separated by Ficoll gradient by the cell bank and preserved into Hanks’ Balanced Salt Solution (HBSS) with 5 percent DMSO in a liquid nitrogen container. The mononuclear cells harvested from a single delivery were placed into five 1 ml cryoprotective vials, at a concentration of 10,000,000 cells per ml. At the time of use, enough vials as needed were rapidly thawed at 37°C in a water bath and gently transferred into a 15 ml centrifuge tube containing either 10 ml of Isolyte S, pH 7.4 (BBraun/McGaw Pharmaceuticals) or HBSS (Gibco). The cells were then centrifuged at the speed of 600g for 15 minutes. The supernatant was discarded and replenished with fresh solution, and re-spun again. This process was repeated three times. Viability of the cells and determination of the number of cells were performed by the trypan blue dye exclusion method. Briefly, 90 µl of trypan blue dye diluted at a concentration of 4 percent was combined with 10 µl of cell suspension. The cells in five quadrants of the hemocytometer were counted and the results averaged. Cell number was adjusted according to the desiderated concentration and a volume of 500 µl of cell suspension was used in each transplant.
2.2.2 Intra-femoral transplantation

On the day of the transplant the animals were anesthetized with isofluorane as described above for the MCAO surgery. The superficial area of the right leg and thigh were shaved and Betadine solution applied. Cutaneous and subcutaneous tissues were carefully dissected using blunt techniques and the femoral vein was isolated. A Hamilton syringe with a 26 gauge needle was employed for the transplant. Once the needle was inserted into the lumen of the femoral vein, the vein was around the needle was ligated. The cells were delivered in a volume of 500 µl over a 5 minute period. After the needle was withdrawn, the ligation was tightened further and the incision closed using a 3-0 silk suture. Afterwards, the anesthesia was stopped, the animal was placed in its cage and observed until awakening. The rats that did not receive a cell transplant received 500 µl the HUCBC-free resuspension solution (Isolyte S, pH 7.4) used in the animals receiving cells.

2.2.3 Intra-penile vein transplantation

Similar to the procedure outlined above, the animals were re-anesthetized with isofluorane. The penis of the animal was carefully exposed and the dorsal vein identified. Betadine solution was applied to the whole exposed surface of the genitals. A 1cc syringe with a 31 gauge needle was employed for the cell transplant. Once the needle was inserted into the lumen of the dorsal vein, the cells were delivered in a volume of 500 µl over a 5 minute period. After the needle was withdrawn, pressure was applied on the vein for approximately 20 seconds. The anesthesia was stopped, the animal was placed in its cage and observed until regaining consciousness. As for the femoral transplants, the
rats that did not receive HUCBC were injected with 500 µl the HUCBC-free resuspension solution (Isolyte S, pH 7.4 or HBSS).

2.3 Animal sacrifice and preparation of tissues

All sacrifice procedures were performed in the Vivarium facility of the College of Medicine (University of South Florida), according to the guidelines of the National Institute of Health approved by the University of South Florida Institutional Animal Care and Use Committee. All rats were anesthetized with pentobarbital (150 mg/kg, intraperitoneally). When animals were non-responsive to toe pinch, a 22 gauge needle was inserted into the left ventricle, the auricola of the right atrium was perforated, and animals were perfused with 0.1 M phosphate buffer (PB) pH 7.4, followed by 4% paraformaldehyde in PB (PFA). Afterward, brain and other organs (heart, lungs, liver, kidney, spleen, thymus) were harvested and placed in 10 fold volume of 4% PFA for 24 hours. At the end of the post-fix period, organs were placed in 20% sucrose in 0.1 M PB for 24 hours and finally cryopreserved at ~80°C.

When perfusion of the animals was not necessary (such as in experiments conducted in Chapter 4 and 5), animals were anesthetized with Isofluorane and decapitated using a standard guillotine.
CHAPTER THREE

DOSE EFFECT OF CORD BLOOD INFUSION ON FUNCTIONAL AND
HISTOLOGICAL RECOVERY OF RATS SUBJECTED TO MIDDLE
CEREBRAL ARTERY OCCLUSION

3.1 Introduction

Several studies have reported that infusion of human umbilical cord blood cells (HUCBC) after the induction of brain ischemia can improve behavioral outcome (Chen, Sanberg et al. 2001; Willing, Lixian et al. 2003; Willing, Vendrame et al. 2003). The seminal findings of Chen et al. (Chen, Sanberg et al. 2001), demonstrated that treatment by intravenous delivery of HUCBC 24 hours after MCAO in rats could improve functional recovery, as evidenced by improvements on the rotarod test and Modified Neurological Severity Score (mNSS). Subsequent studies have shown that the behavioral recovery induced by intravenous HUCBC treatment of MCAO rats is comparable if not better than the recovery observed after intra-cerebral transplantation (Willing, Lixian et al. 2003). These observations have raised great hope and interest in the area of hematological stem cell transplantation therapy for stroke. However, several important questions regarding HUCBC therapeutic potential remain unanswered: 1) what is the best dose for these cells? 2) What is the therapeutic time-window for employment of these cells post-stroke (hours, days, weeks)? 3) is it solely the stem cell population mediating
recovery and 4) what is the therapeutic mechanism (neuron replacement or neuron sparing)?

In this study, we examined the HUCBC therapeutic dose in stroke, as answering this question provides an important platform from which the remaining questions may be addressed. The studies by Chen et al. (Chen, Sanberg et al. 2001) and Willing et al. (Willing, Lixian et al. 2003) have employed HUCBC doses around $10^6$ cells and shown amelioration of stroke-induced functional deficits. Studies using HUCBC in other neurodegenerative diseases models, have shown functional recovery required from relatively few (10,000) up to megadoses (such as $5\times10^7$ cells) of HUCBC (Chen and Ende 2000; Garbuzova-Davis, Willing et al. 2003).

Given the wide dose range in which HUCBC had been reported to be therapeutic, we sought to investigate whether there was an optimal dose at which HUCBC showed efficacy in the MCAO model. In particular, we wanted to determine whether improvement in behavioral outcome measures would show a HUCBC dose dependent effect. Additionally, we measured the infarct volume to evaluate quantitatively whether HUCBC could reduce the amount of tissue damage present after MCAO, as there are no consistent reports in the literature on whether cord blood treatment could ameliorate brain structural and histological alterations induced by MCAO; this may be important features when evaluating the impact of cord blood cell infusion in this disease model.

Although intracerebral transplantation is commonly used to deliver cells for neuronal cell transplantation, the intravenous route has also been demonstrated to be effective when hematopoietic cells (bone marrow or blood derived cells) are used, as these have been detected in the brain parenchyma and shown to induce behavioral recovery in
animal models of neurodegenerative disorders (Garbuzova-Davis, Willing et al. 2003; Mahmood, Lu et al. 2004). Additionally, some investigators have demonstrated that cord blood cells and other types of cells can be chemotactically attracted to the site of injury (Aboody, Brown et al. 2000; Chen, Sanberg et al. 2001). Since HUCBC were exclusively employed through the intravenous route in the current study, we wondered whether these cells could specifically reach and survive in the injury hemisphere 4 weeks post-infusion. Moreover, given the intravenous delivery route and the possible relevance to their therapeutic efficacy we profile the HUCBC distribution in other organ systems.

3.2 Materials and Methods

Animals were divided into seven experimental groups: sham surgery (n=4), MCAO only (n=13), rats infused with $10^4$ (n=6), $10^5$ (n=6), $10^6$ (n=6), $10^7$ (n=6) or 3-5 x$10^7$ (n=4) HUCBC 24 hours after MCAO. After the transplant, all animals (including animals that had not received a cell transplant) were injected with cyclosporin (CSA) in the amount of 10 mg per kilograms of weight of the animal. Since cyclosporin has been shown to be neuroprotective in animal models of stroke (Borlongan, Stahl et al. 1999), it is critical to control for this in any transplant study that administers this drug. Therefore, in our study, all animals regardless of the treatment (HUCBC or media) were injected with cyclosporine. Using this design ensures that any change seen in the cell transplanted animals relative to non-transplanted animals cannot be explained by cyclosporin administration alone. Cyclosporine was diluted in commercially available olive oil at a concentration of 1:1. The injection was repeated daily for the duration of this study. In
order to protect the immunocompromised animals from opportunistic infections, all animals were housed in a vented hood and their drinking water was replaced with acidified water.

3.2.1 Behavior tests

The battery of tests consisted of measurements of spontaneous activity, elevated body swing test, and step test and were performed in all animals before MCAO, and at 2 and at 4 weeks after MCAO.

3.2.1.1 Measurement of Spontaneous activity

Spontaneous activity was measured with the automated VersaMax System (Accuscan Instruments, Columbus, OH, USA). For the daylight spontaneous activity test, rats were placed for 1 hour in a Plexiglas box (35x20x30 cm) with bedding; the box was surrounded by two levels of infrared beams. Thirteen locomotor parameters including horizontal activity (HA), movement number (MVN), movement time (MVT), total distance (TD), vertical activity (VA), vertical movements (VMN), stereotypy number (SN), stereotypy counts (SC), stereotypy time (ST), clockwise rotation (CR), counterclockwise rotation (CCR), center distance (CD) and center time (CT) were evaluated. Activity was recorded every 5 minutes for a total time of 1 hour. The nighttime spontaneous activity test was conducted with the same procedures except that the activity was measured for 12 hours, through the dark phase of the light cycle, and recorded every hour.
3.2.1.2 Step test

This test was performed on a hard flat surface where the rats were placed for a few minutes before the actual test in order to let them habituate to the testing area. The rats were held tightly immobilizing all four paws except either the right or left forelimb. With the free forelimb placed on the surface, they were dragged for a length of 1 meter towards the direction of the placed forelimb. The number of steps made was counted. The same procedure was performed with the other forelimb dragging the animal for the same distance in the opposite direction. The difference between number of steps performed with the right paw and number of steps performed with the left paw was calculated.

3.2.1.3 Elevated Body Swing test

For this test, the animal was placed on a Plexiglass cage with fresh bedding and allowed to acclimate for about 5 minutes before starting the test. Rats were held tightly by the tail at approximately 10 cm above the surface of the testing area. When rats are held as described, they spontaneously lift their body toward the right or the left of their midline body axis. The frequency of left or right swings was counted over 20 consecutive trials. Because it has been observed that prior to undergoing stroke some rats already demonstrate an asymmetry, swinging more towards one specific side, we calculated the elevated body swing test “bias” by taking the absolute value of the difference between the number of swings on the impaired side minus 10, since a nonbiased animal should turn to both sides equally. Therefore, a rat that presents no bias will score 0 and an increasing score represents greater lateral movement with a maximum score being that of 10.
3.2.2 Brain histological assessment

Four weeks after the transplant animals were sacrificed and brain were harvested as described in chapter 2.

3.2.2.1 Nissl-Thionine staining

Cryopreserved brains were cut with a cryostat in 20um thick sections. All sections were preserved at –80°C until use. The Nissl-Thionine dye was obtained by combining 625ml of Thionine with 500ml of a solution prepared by adding 18 ml of 6% glacial acetic solution with 100ml of 1N NaOH. The staining was performed as follows. Cryopreserved slides were allowed to reach room temperature and were subsequently soaked in 100% ethanol for 10 minutes. This step was followed by the immersion of the slides in a decreasing ethanol gradient: 95% ethanol for 2 minutes, 70% ethanol for 2 minutes, 50% ethanol for 2 minutes. Afterwards, the slides were immersed in distilled water for 1 minute and the Nissl-Thionin dye was then applied for 50 seconds. The slides were immediately re-soaked in water for 2 minutes and then dehydrated through an increasing ethanol gradient (50% ethanol for 3 minutes, 70% ethanol for 3 minutes and 95% ethanol for 3 minutes and 100% ethanol for 7 minutes). Finally slides were left in xylene for 15 minutes. Slides were then coversplipped with Permount (ProSciTech).

3.2.2.2 Measurement of Infarct volume

The areas of cerebral infarction were delineated at six pre-selected coronal sections at a distance of 1 mm apart beginning 1.7 mm anterior to bregma through 3.3 mm posterior to bregma. The area of ischemic damage and the total area of the contralateral hemisphere were quantified using an image analyzer (Image Pro Plus). The total volume of ischemic tissue and that of the contralateral hemisphere were calculated as follows: total
volume = \sum \text{(area of predefined coronal sections (mm))} \times 1 - 6 \times \text{(intersection distance (mm))}

where each measured area of the six coronal sections was multiplied by the distance to the subsequent section, and the total volume was obtained by calculating the sum of these measures. Since at one month after MCAO the injured brain hemisphere has typically undergone a shrinkage process that occurs to a variable degree, we found it necessary to normalize the volume of damage for the decreased size of the hemisphere. In order to do so, we calculated the volume of ischemic damage as percent of volume of contralateral (intact) hemisphere. The amount of damage was quantified by taking into account the observable damage (the “necrotic core” and the bystander white tissue as stained with Nissl-Thionine) in addition to the lacunar spaces.

3.2.3 Tracking the intravenous transplanted cells.

The same animals employed in the behavioral and histopathological studies underwent polymerase chain reaction (PCR) analysis for human nested primers and immunohistochemistry analysis. Animals were taken from all seven experimental conditions as described previously. All animals were transplanted intravenously 24 hours after the stroke surgery and sacrificed 4 weeks after the cell transplant, according to the methods described in Chapter 2. Half of the animals per group underwent PCR analysis, the other half underwent immunohistochemical analysis.

3.2.3.1 Polymerase Chain Reaction

3.2.3.1.1 DNA extraction

Brains and other organs (heart, lungs, liver, kidney, spleen, thymus, bone marrow) were sampled. A fragment of approximately 50 µm³ for each sampled organ was
suspended in 500 \( \mu l \) of lysis buffer (400\( \mu g/ml \) of Proteinase K in 20mM Tris-CL, 5mM EDTA, 400mM NaCl, 1\% SDS) left for 12 hours in a water bath at 55\(^\circ\)C. An equal volume of Phenol (Invitrogen) was added to the suspension, the entire mixture was then vortexed and spun for 3 times at 1500g for 10 minutes. The aqueous phase was collected and resuspended in Phenol three times and spun as in the previous step. The same process was repeated once with Chloroform (Gibco). DNA was subsequently precipitated by adding an equal volume of Isopropanol (Sigma). After centrifuging the tube for 5 minutes at 1200g, a DNA pellet was obtained. This was then washed in 70\% ethanol and dried at room temperature for 15 minutes. The pellet was then dissolved in 100\( \mu l \) of Tris EDTA (TE) (pH 8.0) at 37\(^\circ\)C overnight. For each sample, the DNA obtained was quantified using the SmartSpec spectrophotometer set at the wavelength of 260 nm.

3.2.3.1.2 PCR protocol for nested primers

PCR was run for the human glycerol-3-phosphate dehydrogenase gene using the following primers (sense: GGCTGGGACTCATGGAGAT, antisense: CGGGTAAAGTCTGGAG-AAAG) at a concentration of 500nM for 30 cycles. Each cycle was run as follows: 95\(^\circ\)C for 30 sec, 67\(^\circ\)C for 30 sec, 72\(^\circ\)C for 30 sec. A second PCR was run for nested primers (sense: TCTTGGAGAGCTGTGGTGTTG, antisense: GTTACCTGAAAGGACTGC), at a concentration of 500 nM for 30 cycles. Each cycle was run as follows: 95\(^\circ\)C for 2 min, 95\(^\circ\)C for 30 sec, 60\(^\circ\)C for 30 sec, 72\(^\circ\)C for 30 sec. A positive control of human DNA (60pg) and a “non-template” sample were run with each reaction.
3.2.3.1.3 Polyacrylamide gel preparation and Silver staining

DNA products were run on 3.5% polyacrylamide gels. Each gel was prepared by adding 100µl of ammonium persulfate (APS, Biorad) and 5µl of tetramethylethylenediamine (TEMED, Biorad) to a solution composed of 2ml of acrylamide (Biorad), 2ml of TBE (Tris 27gr, Boric Acid 13.75gr and 10ml EDTA) and 6 ml of water. Gels were prepared using the Biorad gel mounting cassettes after 15 minutes of solidification. Samples were run in the amount of 5 µl added to 2 µl of loading buffer. A 100 base pair ladder (New England Biolabs) was run in each gel. After gels were run for 20 minutes at 90 Amps, they were soaked in 10 % ethanol for 3 minutes. Silver staining was performed by immersing the gels in 1% nitric acid for 3 minutes. After rinsing with distilled water, the gels were soaked in 0.1% silver nitrate for 10 minutes in the dark. The gels were then transferred to the developing solution (6g sodium carbonate 120µl formaldehyde in 100ml of water) until the bands appeared. After soaking the gels in 10% acetic acid for 5 minutes, the gels were left in 1 % glycerol for 2 hours. Finally, gels were dried between cellophane films and scanned.

3.2.3.2 Immunohistochemistry Techniques

3.2.3.2.1 Quenching of Autofluorescence

The sections were rinsed 3 times for 10 min with phosphate buffered saline (PBS), and an autofluorescence quenching solution (kindly provided by Dr Ann Steele, All Childrens Hospital, Department of Pathology, St Petersburg, Florida) was applied for 10 seconds at room temperature at a dilution of 1:100 from the given stock concentration.
3.2.3.2 Imunohistochemistry protocol

Primary mouse monoclonal antibody against human nuclei (HuNu, Chemicon, Inc) was applied overnight at a 1:50 dilution. Slides were then rinsed 3 times for 10 min with PBS followed by a two hour incubation in a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Alexa 488, Molecular Probes). Control slides were stained with the same protocol with the omission of the primary antibody. Slides were washed five times in PBS and DAPI staining (Molecular Probes) was performed to visualize nucleated cells. Finally, slides were coverslipped with Vectashield and preserved in the dark at 4°C.

3.2.4 Statistical Analysis

The behavioral test data were analyzed using analysis of variance (ANOVA) with repeated measures. A homogeneity of variance was also applied if this test was significant (p<0.05), the Kruskall-Wallis H-Test was employed for post hoc comparisons. Statistical analysis of the brain infarct measurements was performed with ANOVA.

3.3 Results

3.3.1 Infusion of cord blood cells reduces the stroke-induced hyperactivity.

3.3.1.1 Daytime spontaneous activity test

For eleven of the thirteen analyzed parameters the overall ANOVA revealed significant differences between the groups (p<0.0001 to p<0.04). Moreover, on nine of the thirteen variables there were significant interactions between groups and length of time post
transplant, groups and observational period, and groups, length of time post-transplant
and observational period (p< 0.001 to p< 0.03) (Table 3.1).

For the parameters CCR, HA, MVT, SC, VMN differences between the dose groups were
examined at 2 and 4 weeks (Table 3.2). On CCR, at 2 weeks the animals treated with 10^5,
10^6 and 3-5x10^7 cells were significantly less active than the media-treated controls
(p=0.03, p=0.005, p=0.04 respectively). At 4 weeks, the 10^7 HUCBC group also became
significantly less hyperactive than the stroke-only rats (p=0.03) and the 10^6 and 3-5x10^7
groups remained significantly less active (p=0.008 and p=0.02 respectively). Similarly,
on HA, there was a significant reduction of movement in the 10^5 and 10^6 and 3-5x10^7
groups compared to the non-recipient MCAO rats at 2 weeks (p=0.005 for all three
groups), and at 4 weeks the 10^6 and 10^7 dose groups were significantly less active than the
MCAO only animals (both p=0.05). On MVT, at 2 weeks post-transplant the animals in
the 10^4, 10^5 and 10^6 groups were significantly less active than the controls (p=0.009,
p=0.043, p=0.02). At 4 weeks the only group that was significantly less active was the
10^7 group (p=0.05). On SC there was a reduction in hyperactivity only at 2 weeks for the
group 10^6 (p=0.04). On VMN there were no differences between the treated groups and
the controls, but there was a significant reduction of the activity between the 10^5 dose
group and the 3-5x10^7 group (p=0.010). On CR, TD and VA there were no interactions so
we examined the differences between groups regardless of the time post-transplant or the
observational period (Table 3.2). On CR, rats that had been treated with 10^5 and 10^6 cells
were less active than the stroke-only controls (p=0.046 and p=0.005 respectively). In
addition, animals that received 10^6 cells were significantly less active than animals in the
Table 3.1

Overall ANOVA for Daytime Spontaneous Activity: p value, F ratio, degrees of freedom

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Group x time post-infusion</th>
<th>Group x observational period</th>
<th>Group x time post-infusion x observational period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counterclockwise rev (CCR)</td>
<td>0.003</td>
<td>0.0902</td>
<td>&lt;0.0001</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>4.05</td>
<td>0.35</td>
<td>3.27</td>
<td>1.53</td>
</tr>
<tr>
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<td>6.38</td>
<td>6.38</td>
<td>66.418</td>
<td>66.418</td>
</tr>
<tr>
<td>Center time (CT)</td>
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<td>0.198</td>
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<td>&lt;0.0001</td>
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<td>Center distance (CD)</td>
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<td>0.743</td>
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<td>6.38</td>
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<td>66.418</td>
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<td>Clockwise rotation (CR)</td>
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</tr>
<tr>
<td>Horizontal activity (HA)</td>
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<td>0.127</td>
<td>0.150</td>
<td>0.033</td>
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<td>1.19</td>
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<td>66.418</td>
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<td>&lt;0.0001</td>
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<td>66.418</td>
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<td>Movement time (MVT)</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td>&lt;0.0001</td>
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<td>66.418</td>
</tr>
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<td>0.063</td>
<td>0.001</td>
<td>&lt;0.0001</td>
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<td>66.418</td>
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<td>6.38</td>
<td>6.38</td>
<td>66.418</td>
<td>66.418</td>
</tr>
<tr>
<td>Vertical activity (VA)</td>
<td>0.027</td>
<td>0.598</td>
<td>&lt;0.0001</td>
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</tr>
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<td>2.70</td>
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<td>6.38</td>
<td>6.38</td>
<td>66.418</td>
<td>66.418</td>
</tr>
<tr>
<td>Vertical movements (VMN)</td>
<td>&lt;0.0001</td>
<td>0.127</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
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<td>8.58</td>
<td>1.79</td>
<td>2.68</td>
<td>4.94</td>
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<td></td>
<td>6.38</td>
<td>6.38</td>
<td>66.418</td>
<td>66.418</td>
</tr>
<tr>
<td>Stereotypy time (ST)</td>
<td>0.030</td>
<td>0.0005</td>
<td>0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
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<td>2.63</td>
<td>5.24</td>
<td>1.69</td>
<td>3.01</td>
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<td>66.418</td>
<td>66.418</td>
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</table>
Table 3.2
Post-hoc Statistical Comparison for Daytime Spontaneous Activity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time of analysis</th>
<th>Significance (p and $\chi^2$ between the media treated and HUCBC treated groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counterclockwise rev (CCR)</td>
<td>2-weeks</td>
<td>$10^7$ (p=0.03, $\chi^2_i=4.43$), $10^6$ (p=0.005, $\chi^2_i=7.87$), 3-5x10^7 (p=0.04, $\chi^2_i=4.15$)</td>
</tr>
<tr>
<td></td>
<td>4-weeks</td>
<td>$10^6$ (p=0.008 $\chi^2_i=6.92$), $10^5$ (p=0.03 $\chi^2_i=4.43$), 3-5x10^7 (p=0.02 $\chi^2_i=5.12$)</td>
</tr>
<tr>
<td>Clockwise rotation (CR)</td>
<td>2 and 4-weeks</td>
<td>$10^7$ (p=0.046 $\chi^2_i=3.97$), $10^6$ (p=0.005 $\chi^2_i=7.81$)</td>
</tr>
<tr>
<td>Horizontal activity (HA)</td>
<td>2-weeks</td>
<td>$10^7$ (p=0.05 $\chi^2_i=3.72$), $10^6$ (p=0.05 $\chi^2_i=3.72$), 3-5x10^7 (p=0.05 $\chi^2_i=3.86$)</td>
</tr>
<tr>
<td></td>
<td>4-weeks</td>
<td>$10^6$ (p=0.05 $\chi^2_i=3.72$), $10^5$ (p=0.05 $\chi^2_i=3.72$)</td>
</tr>
<tr>
<td>Movement time (MVT)</td>
<td>2-weeks</td>
<td>$10^5$ (p=0.009 $\chi^2_i=6.69$), $10^4$ (p=0.043 $\chi^2_i=4.06$), $10^5$ (p=0.020 $\chi^2_i=5.40$)</td>
</tr>
<tr>
<td></td>
<td>4-weeks</td>
<td>$10^7$ (p=0.05 $\chi^2_i=3.72$)</td>
</tr>
<tr>
<td>Stereotypy counts (SC)</td>
<td>2-weeks</td>
<td>$10^6$ (p=0.04 $\chi^2_i=4.06$)</td>
</tr>
<tr>
<td></td>
<td>4-weeks</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total distance (TD)</td>
<td>2 and 4-weeks</td>
<td>$10^4$ (p=0.04 $\chi^2_i=3.91$), $10^3$ (p=0.04 $\chi^2_i=4.03$), $10^6$ (p=0.001 $\chi^2_i=9.76$), $10^5$ (p=0.046 $\chi^2_i=3.97$), 3-5x10^7 (p=0.05 $\chi^2_i=3.71$)</td>
</tr>
<tr>
<td>Vertical activity (VA)</td>
<td>2 and 4-weeks</td>
<td>$10^7$ (p=0.002 $\chi^2_i=9.27$)</td>
</tr>
<tr>
<td>Vertical movements (VMN)</td>
<td>2-weeks</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>4-weeks</td>
<td>between $10^4$ and 3-5x10^7 (p=0.010 $\chi^2_i=6.54$)</td>
</tr>
</tbody>
</table>
$10^4$ dose group (p=0.056). Similarly, on TD, where a significant activity reduction was present in all dose groups ($10^4$ for p=0.04, $10^5$ for p=0.04, $10^6$ for p=0.0018, $10^7$ for p=0.046 and $3-5 \times 10^7$ for p=0.05), there was more activity reduction in the $10^6$ group compared to the $10^4$ group (p=0.05). On VA, where the $10^5$ group was less active than MCAO only (p=0.002), the group $10^6$ was less active than the $10^4$ group and the $10^5$ group (p=0.02 and p=0.05). In Figure 3.1 we present the total activity at 2 and 4 weeks (expressed as percent of baseline) for two representative parameters (CCR and HA). Figure 3.2 presents the activity of all rats measured with the same two parameters, but illustrated along the entire observational period of the test (60 minutes).

### 3.3.1.2 Night-time spontaneous activity test

Nighttime spontaneous activity was tested during the night at intervals of 1 hour for a period of 13 hours. The overall ANOVA revealed significant differences between the groups for eleven of the thirteen examined variables, with significances ranging from p<0.0001 to p=0.05. Differences in activity among the groups were also present across time (2 and 4 weeks) and at the different time points (13 measures in 13 hour test) for all the measures except for CR, MVN and SN (p<0.0001 to p=0.0025) (Table 3.3). The Kruskall-Wallis H-Test was employed for post hoc analysis. A significant decrease in activity of animals in the treated groups was found only in HA (dose of $10^5$ for p=0.035).
Figure 3.1
Daytime spontaneous activity for two representative parameters of movement: counterclockwise rotations (CCR) (A) and horizontal activity (HA) (B). Data are expressed as percent of baseline activity. For CCR: *p<0.05 at 2 weeks and #p<0.05 at 4 weeks. For HA: *p<0.005 at 2 weeks and # p=0.05 at 4 weeks.
Figure 3.1
Daytime spontaneous activity for two representative parameters of movement: counterclockwise rotations (CCR) (A) and horizontal activity (HA) (B). Data are expressed as percent of baseline activity. For CCR: *p<0.05 at 2 weeks and #p<0.05 at 4 weeks. For HA: *p<0.005 at 2 weeks and # p=0.05 at 4 weeks.
Table 3.3
Overall ANOVA for Nighttime Spontaneous Activity: p value, F ratio, degrees of freedom

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Group x time post-transplant</th>
<th>Group x observational period</th>
<th>Group x time post-transplant x observational period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counterclockwise rev (CCR)</td>
<td>0.058</td>
<td>0.008</td>
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<td>&lt;0.0001</td>
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<td>72</td>
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<tr>
<td>Center time (CT)</td>
<td>0.0001</td>
<td>0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>6.61</td>
<td>4.72</td>
<td>5.57</td>
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<td></td>
<td>6.38</td>
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<tr>
<td>Center distance (CD)</td>
<td>0.029</td>
<td>0.014</td>
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<td>&lt;0.0001</td>
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<td></td>
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<tr>
<td>Clockwise rotation (CR)</td>
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<td>0.264</td>
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<td>Horizontal activity (HA)</td>
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<tr>
<td>Movement time (MVT)</td>
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<td>&lt;0.0001</td>
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<td>Stereotypy number (SN)</td>
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<td>0.717</td>
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<td>Stereotypy counts (SC)</td>
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<td>Total distance (TD)</td>
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<td>Vertical movements (VMN)</td>
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<td>0.006,38</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1.35</td>
<td>3.55</td>
<td>3.69</td>
<td>7.37</td>
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<td></td>
<td>6.38</td>
<td>6.38</td>
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<tr>
<td>Stereotypy time (ST)</td>
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<td>0.0005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>11.44</td>
<td>3.10</td>
<td>4.14</td>
<td>3.67</td>
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<tr>
<td></td>
<td>6.38</td>
<td>6.38</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>
3.3.2 Infusion of high doses of cord blood cells induces a complete recovery of asymmetry deficits measured with the Step test.

The normal rats took the same number of steps with the right and the left paw during all tests. As shown in Figure 3.3 the group of rats that was stroked and did not receive a HUCB transplant displayed an increase in asymmetry (expressed as percent of baseline difference between right and left steps) of about 240% and 200% at 2 and 4 weeks, respectively. Groups that received the higher doses of cells such as $10^7$ and $3-5\times10^7$ showed significantly less asymmetry than the non-treated group ($p=0.01$ and $p=0.02$) at both 2 and 4 weeks. Interestingly, at 4 weeks both these groups were performing at pre-stroke baseline levels. Additionally, at this late time point the animals of the $10^5$ groups also presented with a significant reduction of asymmetry compared to the animals in the non-treated group ($p<0.05$).

3.3.3 Cord blood infusion reduces long-term deficits in the Elevated Body Swing Test.

Prior to MCAO all animals had a bias of less than 2. At 2 weeks post-transplant, there were no significant differences between dose groups and stroked animals that had not received a cell infusion. However at 4 weeks, the bias of the animals treated with the dose of $10^6$ and $3-5\times10^7$ was significantly less than MCAO control rats ($p=0.005$ and $p=0.04$ respectively) (Figure 3.4).
Figure 3.3
Step test: Expressed as percentage of asymmetry from baseline (pre-MCAO or week 0): Performance on this task was assessed at the two week timepoint and four week time-point; HUCBC dose dependent recovery was found to be significant as indicated by #p<0.05 and *p<0.01.
Figure 3.4
Elevated Body Swing Test (EBST): percent of baseline performance for all experimental groups indicated in abscissa. #p=0.04 for the 10(6) group and #p=0.005 for the 3-5x10(7) group.
3.3.4 Cord blood infusion induces histological benefit in brains of rats subject to MCAO.

In the infarcted brain examined at one month after the stroke, four distinct phenomena were apparent. First, at one month after the MCAO surgery the injured hemispheres had shrunk. Secondly, as shown in Figure 3.5, there was also a loss of tissue (observable across all bregma levels) due to the liquefaction process that follows the edema and necrotic events typically observed within the first week post-stroke. Four weeks after the ischemic injury most of the tissue involved in this process has been lost and substituted by empty spaces (generally named “lacunae”). This feature was mostly evident in the most caudal sections examined. Thirdly, within the striatum and cortex there was an extensive loss of cells, with a complete loss of cytoarchitecture. Four, in most of the MCAO brains it was possible to identify the “necrotic core”, a well-defined area that stained more insensitively with the Nissl-Thionine dye (representing the area of irreversible cell damage). In the brains of MCAO rats that had received at least $10^6$ cells, the process of shrinkage was evident, together with the variable presence of the necrotic core and scattered lacunar spaces. On the contrary, the loss of cytoarchitecture in striatum and cortex was less dramatic, almost negligible in some brains. Figure 3.6 shows some of these histological characteristics in detail.

3.3.5 Cord blood infusion induces reduction of infarct volume in MCAO brains

The infarct volume was quantified as explained in Material and Methods section at six predefined coronal sections. Figure 3.7 presents a picture of the six coronal sections
Figure 3.5
a, b and c illustrate coronal sections of brains of MCAO rats stained with Nissl-Thionine. Bregma levels are indicated in the lower left corner. Star symbol indicates lacunar spaces. Inserts show the necrotic core and the loss of architecture.
Figure 3.6
Coronal sections of brains of MCAO rats treated with HUCBC. Numbers in lower left corners indicate the bregma levels.
*=lacunar spaces, #=necrotic core, ##=loss of cytoarchitecture.
d and f: treated with 10(7) cells; g and e: 3-5x10(7) cells; h= treated with 10(6) cells. Boxed area indicate area of similar pathology.
Figure 3.7
Representative Nissl-Thionine staining of brain coronal sections (bregma level indicated), 4 weeks after MCAO and treatment with media or HUCB cells (3-5x10⁷).
(from bregma 1.7 to bregma –3.3) stained with Nissl-Thionine of two representative brains treated with no cells (media) and with 10⁷ HUCBC. In addition to a reduction in histological anomalies observed with HUCBC infusion after MCAO, there was also a reduction in the amount of damage or infarct size as a consequence of our treatment. The administration of HUCBC at the dose of 10⁷ but not at lower dose could significantly reduce the volumes of ischemic brain damage (Figure 3.8). Infarct volume in the stroked groups was 33.15 ± 4.29% of the volume of contralateral hemisphere, while animals of the 10⁷ doses showed infarcts involving only 11.46 ± 4.13% of the hemisphere. Animals that had received a higher dose of cells was 20.61 ± 4.16% of the contralateral hemisphere. As shown in Figure 3.8, groups injected with other doses also had a decreased infarct volume, dependent on the amount of cells infused. However differences between the infarct volumes of these groups and the controls were not statistically significant.

3.3.6 Cord blood cells are detected in spleen and right brain hemisphere with nested PCR for human primers.

Right and left brain hemispheres, heart, lung, liver, spleen, thymus, kidney and bone marrow were sampled. In animals of the lowest dose groups such as the 10⁴ and 10⁵ cell groups no human DNA was found in any organ. In animals injected with 10⁶, 10⁷ and 3-5x10⁷ cells, human DNA was found in spleen, right brain hemisphere (injured), and heart (Figure 3.9). Human DNA was never detected in other organs such as thymus, liver, bone marrow, kidney and lung.
Figure 3.8
Quantification of the ischemic brain injury volume, across all treatment groups indicated in abscissa (*p<0.05).
Figure 3.9
PCR detection of the human DNA gene in the organs (t=thymus; Li=liver; bm=bone marrow; sp=spleen; lb=left brain; rb=right brain; h=heart; Lu=lung) of MCAO rats 4 weeks after cord blood transplantation (doses indicated to the right of gels). HUCBC doses are indicates on the left of each gel. M=marker; fp=1st pcr product; nc=negative control; pc=positive control.
3.3.7 Immunochemical labeling of human cells with an antibody against Human Nuclei confirms the PCR results.

There was extensive autofluorescence present in spleen, heart, liver and kidney. The quenching solution reduced the autofluorescence in all organs examined except in lung, heart and thymus where the quenching solution decreased the interfering signal only minimally. Human nuclei immunoreactive cells were detected in spleen and right brain hemisphere of animals injected with $10^6$, $10^7$ and $3-5 \times 10^7$ cell doses (Figure 3.10). Here the immunopositive cells seemed to be localized within blood vessels or at the perivascular space. Few cells were detected in the parenchyma of either spleen or brain. In liver and kidney no HUCBC were found.

3.4 Discussion

The reduction of stroke-induced hyperactivity is considered a specific and sensitive measure for the evaluations of experimental treatments in the MCAO rats. Here we found that HUCBC doses equal to or greater than $10^6$ were able to induce significant activity reduction when this was measured at 2 weeks. Doses higher then $10^6$, such as $10^7$ and $3-5 \times 10^7$, could induce a further improvement that could be also observed at 4 weeks. At lower doses ($10^4$ and $10^5$) although there was a similar tendency this reached significance only for certain measures.

When the Step test was performed after the employment of this array of HUCBC doses, only the highest doses had a significant effect. Doses of $10^7$ and $3-5 \times 10^7$ were able to revert the motor ability of the rats to levels comparable to normal rats.
Figure 3.10
Immunofluorescent detection/localization of HUCBC by an anti-human nuclei antibody (HuNu) in spleen (a,b) and brain (c,d,e). Arrows indicate positive immunolabeling.
Although the spontaneous activity test and the Step test demonstrated an effect of HUCBC infusion at 2 weeks after transplantation, the elevated body swing test did not show any significant improvement at this early time point. However, at 4 weeks after transplant rats injected with $10^6$ and 3-5$x10^7$ cells displayed a significantly decreased bias compared to the media-receiving rats. Curiously, the $10^7$ dose group did not demonstrate any statistical improvement as shown by the Spontaneous Activity and Step Test for this group. However, review of individual performance on the EBST suggested the presence of two outliers (>2.5 S.D. from mean); when these were excluded from the analysis, there was statistically significant improvement on this test as well (p<0.031).

We can conclude from these observations that the mitigation of MCAO induced deficits depends on the amount of HUCBC injected. Specifically, when $10^6$ or higher amounts of HUCBC are employed after permanent MCAO there is significant recovery in behavioral performance; however, at the highest HUCBC doses employed ($10^7$ and 3-5$x10^7$) recovery of behavioral function shows a plateau effect showing no significant improvement over that of the $10^7$ dose.

It is also important to mention that these three different behavioral tests have not always shown improvements in function at the same cell dose levels. For instance, the asymmetry analyzed with the Step test shows an amelioration in rats treated with $10^5$ cells when this the test is performed at 4 weeks; at the same time point in the elevated body swing test the group of $10^5$ does not perform better the non-treated animals. Similarly, the $10^7$ group shows an optimal performance in the step test at 2 and 4 weeks, while at both these time points this group fails in displaying a therapeutical benefit with
the elevated body swing test. This observation points out the importance of using an entire battery of tests to evaluate the efficacy of a treatment, especially when utilizing animal models as intrinsically variable such as the middle cerebral artery occlusion in the rat.

MCAO rats that did not receive HUCBC treatment showed extensive neuronal loss in the cortex and striatum of the ischemic hemisphere, while histological analysis of the ischemic brain hemisphere from HUCBC treated animals revealed that histological benefit when doses of $10^6$ HUCBC or higher were infused. However, doses of $10^6$ cells were not able to induce a statistically significant reduction of infarct volume. A significant reduction of infarct size was only observable with doses of $10^7$ and $3-5\times10^7$. Interestingly, these results show that while behavioral benefit can be observed after treatment with $10^6$ cells, only doses of $10^7$ or higher can induce a significant reduction of amount of ischemic damage. While there are studies that have shown histological amelioration in the MCAO brain paralleling behavioral recovery (Obana, Pitts et al. 1988), a number of studies employing the rat MCAO model of stroke have previously described a lack in correlation between improved behavior test performance and brain histology (Grotta, Pettigrew et al. 1988; Wahl, Allix et al. 1992). In particular, Grotta et al. have shown beneficial effect of neuroprotective drugs in improving memory in the absence of histological benefit (Grotta, Pettigrew et al. 1988) and Wahl et al. reported no correlation between performance in neurobehavioral tests (neurological examination, passive avoidance task, and spontaneous activity) and size of infarcts induced by MCAO (Wahl, Allix et al. 1992). This lack in correlation between the histological grade and the
behavioral outcomes raises a number of considerations especially since it seems at odds with the expectation of greater cerebral damage manifesting as worsening behavioral performance. Firstly, there may be compensatory changes in function in other brain regions. At cellular level, surviving cells may compensate for the function of lost neurons, providing “function” to an otherwise damaged brain areas. Second, given the heterogeneous architecture of the brain correlation between pathology observed and behavioral outcome should depend more on the uniqueness of the function to particular brain areas than strictly to infarct volume. Third, different measures of behavioral outcome may have different sensitivity in the detection of a change of infarct volume.

Investigations on the localization of intravenously delivered cells led to the conclusion that HUCBC reached the injured brain and very likely migrated to the spleen. The nested PCR analysis permitted the detection of the HUCBC DNA with high sensitivity, allowing the detection of less than 10 HUCBC cells within the DNA contained in almost 40,000 cells of the sampled organ. Confirmation of this finding with immunohistochemistry using an antibody against human nuclei allowed a morphological and spatial analysis. It was very surprising to find the injected cells in specific organs and not scattered throughout the body. Specifically, brain and spleen seemed to be the target organs. Of even greater interest is the fact that the HUCBC cells were only found in the injured brain hemisphere. Another important finding is the presence of cells in the spleen, which suggests a tropism or migration of these cells to this organ. Similar finding has been reported by other investigators who have employed HUCBC intravenously in models of neurodegenerative diseases. For example, Garbuzova-Davis et al. injected 10,000
HUCBC in mouse model of amyotrophic lateral sclerosis and found 10 weeks post-
injection plenty of transplanted cells homing the spleen (Garbuzova-Davis, Willing et al. 
2003). The HUCBC preparation consists mainly of immature lymphocytes and 
monocytes, which may possess innate homing abilities for primary or secondary immune 
organs. However, when we looked at the primary immune organs such as the thymus and 
the bone marrow no HUCBC were found. These findings seem to strongly support a 
phenomenon of tropism of HUCBC exclusively for the spleen. 
Finally, human DNA was also localized in the heart of two rats injected with doses of $10^7$ 
and $3.5 \times 10^7$. The significance of this finding for physiopathological and therapeutic 
properties of HUCBC remains in question. Either some animals had a heart injury and 
chemoattractants drew HUCBC to an injury site, or HUCBC were trapped in the fine 
vessels of the coronary circulation of the cardiomuscular structure.

In conclusion, we have shown that HUCBC can induce functional and histological 
benefits in MCAO rats in a dose dependent manner. Moreover, at one month after the 
intravenous transplant HUCBC were mainly localized in the injured brain and in the 
spleen. These findings set the foundation for our following investigations directed to 
understanding how HUCBC exert these protective actions.
CHAPTER FOUR

EXAMINATION OF THE EFFECT OF HUCBC INFUSION ON STROKE-INDUCED ALTERATIONS OF SPLENOCYTE TYPE AND FUNCTION

4.1 Introduction

CNS ischemic injury induces a local inflammatory response characterized by activation of resident and recruited cells (such as microglia and infiltrating leukocytes) that produce various pro-inflammatory molecules implicated in the mediation of neuronal damage (Dirnagl, Iadecola et al. 1999). Studies in both the clinical setting and in animal models of stroke have demonstrated that there are alterations in the peripheral immune status, which appear to follow the CNS cascade of pro-inflammatory events (Intiso, Zarrelli et al. 2004; Smith, Emsley et al. 2004). The CNS and the immune system respond to this pro-inflammatory state with a complex molecular and cellular inter-play, mediated in part by the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (Woiciejchowsky, Schoning et al. 1999). The release of glucocorticoids and catecholamines that follows the activation of these respective systems mediate the production of anti-inflammatory interleukins (such as IL-10) from resident microglia and infiltrating monocytes, which provide inhibitory feedback decreasing the stroke-induced pro-inflammatory CNS response. This inhibitory signal is thought to also induce an
immunosuppressive state, which clinical studies have suggested is responsible for the infectious complications seen in the post-acute phase of stroke (Prass, Meisel et al. 2003). The complex changes in the immune system related to brain injury have also been characterized as alterations in the cellular composition and function of peripheral lymphoid organs, such as spleen, thymus and lymph nodes (Prass, Meisel et al. 2003). For example, MCAO in the rat provokes a decrease in the absolute number of splenocytes with a concomitant increase in the ratio of CD4+/CD8+ T-lymphocytes (Gendron, Teitelbaum et al. 2002). In the same study, mitogen proliferation assays for B and T-cells showed a higher proliferative capacity for these cells when harvested from the spleen of stroked rats. Moreover, the cellular and functional changes occurring in these peripheral lymphoid organs is mirrored in the ischemic CNS by a peak in infiltrating T-lymphocytes as well as activated microglia. The importance of these invading leukocytes in stroke induced CNS injury has been shown by the reduction of ischemic volume in mice deficient in the leukocyte adhesion molecule, CD11a/CD18 (Soriano, Coxon et al. 1999). In several rodent models of brain injury, CNS-specific autoreactive T-cells have been shown to either rescue neurons from inflammation related injury (Chen, Ruetzler et al. 2003) or enhance neurotoxicity depending on the subtype of T-cell (Wolf, Fisher et al. 2002). Interestingly, splenocytes derived from rats tolerized with CNS myelin antigens such as myelin basic protein were able to mediate protection against ischemic brain injury when adoptively transplanted into naïve rats (Becker, McCarron et al. 1997; Becker, Kindrick et al. 2003). Numerous reports have now demonstrated that systemic infusion of umbilical cord blood can attenuate the neurobehavioral deficits seen after MCAO surgery in rodents (Chen,

However, the mechanism(s) by which umbilical cord blood mediates this beneficial effect remains unclear. One putative mechanism is that the CD34+ stem cell population (constituting about 1% of mononuclear fraction of umbilical cord blood) transdifferentiate into neurons, once within the injured brain parenchyma (Sanchez-Ramos, Song et al. 2001; Bicknese, Goodwin et al. 2002). However, the detection of these cells in brain injury models when delivered either locally or intravenously has not provided convincing evidence that the cord blood cells survive long-term, let alone replace lost cells (Willing, Lixian et al. 2003). It has been recently shown that intravenously delivered HUCBC not only migrate to the injured CNS in the various models of neurodegeneration but that they can also be found in peripheral tissues including the blood and spleen (Garbuzova-Davis, Willing et al. 2003).

In the previous chapter we showed that the infused cord blood cells were also detected in the spleen. Moreover, the mononuclear fraction of cord blood is mainly composed of immature lymphocytes (see Chapter 1) which, as discussed also in the previous chapter, may possess tropic properties for lymphoid organs.

Given these observations, we have hypothesized that the beneficial improvements seen in our and previous work following HUCBC infusion may be a result of cord blood modulation of peripheral immune alterations induced by the stroke. To test this hypothesis, we examined phenotypic and functional characteristics of spleen cells after stroke and the subsequent infusion of cord blood cells.
4.2 Materials and Methods

Adult male Sprague Dawley rats were randomly assigned to the following experimental groups: sham surgery (n = 6), MCAO only (n = 6) or MCAO plus HUCBC (n = 6).

MCAO surgery was performed as previously described in Section 2.1. Rats received a transplant of 10,000,000 cord blood cells into the penile vein 24 hours after the stroke surgery (see General Materials and Methods, section 2.2.3). One day after HUCBC infusion, all rats were anesthetized with isoflurane and killed by decapitation. Spleens were harvested and dissected free from the surrounding adipose tissue. The net weight of each spleen was measured with the use of the Chaus Scout II scale. Afterwards, each spleen was separated into two parts, one of which was immediately immersed in Trizol reagent for RNA extraction, and the other one was used for preparing single cell suspensions for culture experiments and flow cytometry analysis.

For examination of correlation of spleen weight with infarct volume, an independent set of animals was used. Twelve adult male Sprague Dawley rats were randomly assigned to the following experimental groups: sham surgery (n = 4), MCAO only (n = 4) or MCAO plus HUCBC (n = 4). MCAO surgery and HUCBC transplant (10,000,000 cells) were performed as for the other experiments in this chapter. One day after HUCBC infusion, the rats were killed by decapitation and the brain was quickly removed, sectioned and stained with 2,3,5-triphenyltetrazolium chloride (TTC).
4.2.1 2,3,5-triphenyltetrazolium chloride (TTC) staining and infarct volume quantification.

Brain 6x2-mm coronal slices were prepared with a brain matrix (Aster Industries, Inc). The brain sections were stained with 2% TTC at 37°C for 15 minutes. The TTC stained viable brain tissue dark red, whereas infarcted tissue was unstained. After staining, the sections were fixed in phosphate-buffered formalin (10%) at 4°C followed by scanning on a 1200-dpi UMAX scanner. The border between infarcted and non-infarcted tissue was outlined with the image analysis system Image J (ImageJ 1.31v; http://rsb.info.nih.gov/ij; Wayne Rasband, National Institutes of Health, Bethesda, MD). Briefly, on each slice the unstained area (ischemic brain) was delineated and the infarct volume was calculated according to the thickness of 2 mm per slice. Assessment of infarction size was made in duplicate and mean values were calculated. The infarct areas on each slice were summed and multiplied by slice thickness to give the infarct volumes. To avoid overestimation of the infarct volume the infarcted volume was related to the total volume of the brain to calculate % infarcted volume (that is, [volume of infarcted hemisphere/ total volume of brain]*100).

4.2.2 Detection of RNA expression of cytokine genes

4.2.2.1 RNA extraction

RNA was isolated employing the Trizol reagent (Invitrogen). Tissue samples were incubated with 1ml of Trizol for 5 minutes at room temperature to permit the full tissue dissociation. Chloroform was then added at a concentration of 0.2 ml per 1ml of Trizol Reagent. Tubes were vortexed for 15 seconds and incubated at room temperature for 3
minutes. Samples were then spun at 1,000g for 15 minutes at 4°C. Following the centrifugation step, the solution separated into a lower red (phenol-chloroform phase), an intermediate phase, and an upper aqueous phase (colorless) which contains the RNA. This phase was then transferred to another tube containing 500µl of isopropyl alcohol. Samples were left at room temperature for 10 minutes and then centrifuged at 1,000g for 10 minutes at 4°C. Supernatant was removed and the RNA pellet washed once with 70% ethanol. The RNA pellet was then dried and re-dissolved in RNase-free water (Gibco).

4.2.2.2 RNase Protection Assay

All procedures for the RNase protection assay were conducted at James A. Haley Veterans research center under the supervision of Dr. Carmelina Gemma. All procedures were performed with strict observation of the regulations set by the James A. Haley Veterans research center committee for manipulation of radioactive material. Twenty µg of total RNA from each sample was hybridized with antisense, radiolabeled probes, after which free probe and remaining single-stranded RNA were digested with RNase A/T1. Double-stranded RNase-protected fragments were resolved on 5% denaturing polyacrylamide gels. The probe template used was purchased from Pharmingen (San Diego, CA) and included rat specific sequences for IL-1alpha, IL-1beta, TNFbeta, IL-4, IL-6, IL-10, TNFalpha, IL-2, and interferon (IFN) gamma. A positive control transcript was made using a probe specific for the ribosomal protein L32, housekeeping gene, in order to calculate the specific activity and achieve a sufficient excess of probe over target for L32. The L32 probe was then added to the probe template before the hybridization reaction started. Yeast transfer RNA and rat mRNA were used as negative and positive controls, respectively. Dried gels were placed on a phosphorimager.
screen for 16–20 hours. The phosphorimaging screen was subsequently scanned with a phosphorimager (Molecular Image System GS-363; Bio-Rad, Hercules, CA). The images were processed using Molecular Analyst software (Bio-Rad). The intensity of a band in the computer generated image is directly proportional to the amount of radioactivity within the band. The optical density (OD) values obtained from each band were normalized against the OD obtained from the L32 band in that sample by the following expression: (OD of the sample band / OD of the L32 band X 100).

4.2.3 Splenocyte isolation and cultures

Fresh spleen tissue was homogenized using Stomacher plastic bags (Seward). About 5 minutes of homogenization were necessary to dissociate the spleens into a homogeneous cell suspension. The suspension was collected into 15 ml tubes and settled on ice for 10 minutes. Afterwards, the supernatant was transferred into a new tube and centrifuged at 1,500g for 5 minutes. The supernatant was then removed, the cell pellet was resuspended into 10 ml of red blood cell lysis buffer (Gentra systems) and mixed. The suspension was then centrifuged for 5 minutes and washed once with HBSS/10% heat-inactivated fetal bovine serum (FBS). Each pellet was resuspended in HBSS, passed through a 80-um nylon cell strainer (Fisher Scientific), and washed another time with HBSS. Viable cells were counted using the trypan blue dye exclusion method, an aliquot was set aside for flow cytometry analysis and the rest of the samples were resuspended (about 10^6 cells/ml) in RPMI medium (supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 g/ml streptomycin sulfate, 1.5 mM -glutamine). Splenocytes were
cultured into 96-well plates at 1 x 10^5 cells per well, and plates were incubated at 37 °C and 5% CO₂.

4.2.4 Proliferation assay
Mitogens included lipopolysaccaride (LPS, Sigma) at a concentration of 1 µg/ml for stimulation of B lymphocytes, and Concavalin-A (Con-A, Sigma) at a concentration of 2.0 µg/ml for stimulation of T lymphocytes. Cell suspensions were added in quadruplicate to flat-bottom 96-well plates at a volume of 100µl/well, and plates were incubated at 37 °C and 5% CO₂. After 72 h, the cell proliferation assay was performed. Briefly, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)tetrazolium substrate solution (Sigma, 5 mg/ml in PBS) was prepared and 20 µl was added to each well of the 96-well plates. Living cells convert this substrate to formazan, producing a brown precipitate. The plates were incubated at 37 °C and 5% CO₂ for 3 hours, after which, the supernatant was gently removed using a multichannel pipette and adherent cells were solubilized in 200 µl of 100% DMSO. The resulting color changes were quantified by obtaining optical density (O.D.) readings at 570 nm using the Spectramax Plus spectrophotometer.

4.2.5 Immunophenotyping of spleen cells
Specific leukocyte subtypes were measured by immunofluorescent antibody staining and flow cytometry analysis was performed on a FACScan (BD Biosciences) using CellQuest software. T cells were identified using mouse anti-rat (CD4 and CD8) from BD-Pharmingen (San Diego, CA). Briefly, cells were re-suspended in 200 µl of flow
cytometry buffer (0.02% BSA, 0.05 mM EDTA in PBS) containing Fc-block™ (BD Pharmingen) to reduce non-specific antibody binding. Cells were then incubated in the dark with the appropriate FITC- conjugated antibody (50 µg/mL) for 60 minutes on ice. Afterwards, cells were washed three times with 500 µl flow cytometry buffer and flow cytometry was performed on the FACScan system. Data were analyzed using CELLQuest software (Becton Dickinson).

4.3.6 Cytokine Enzyme-linked immunoadsorbent assays

Cell-free supernatants from Con-A treated splenocytes were collected and stored at –70°C until analysis. IFN-gamma and IL-10 cytokine levels in the supernatants were examined using specific enzyme-linked immunosorbent assay (ELISA) kits (Biosource International) in strict accordance to the following protocols.

4.3.6.1 Quantification of sample protein amounts

Protein concentration in each sample was measured with the BioRad Assay. The BioRad assay dye was diluted in distilled water at a concentration 1:5. Standards were prepared for concentration of 0, 2, 4, 6, 8, 10µg. Samples were diluted 1:5000 in the BioRad Assay and transferred to a glass cuvette. Samples were read on the spectrophotometer Spectramax Plus at 595 nm wavelength.

4.3.6.2 Enzyme-linked immunoadsorbent assays

IFN-gamma and IL-10 cytokine levels in the supernatants were examined using specific ELISA kits obtained from Biosource International. The amount of 50 µL of standard and samples were loaded into the appropriate wells together with 50 µL of Incubation Buffer. The plate was incubated at 4°C overnight. Afterwards, wells were washed with Working
Wash Buffer four times. The Biotin conjugated antibody was then added to each well (100µl per well) and the plate was incubated for 2 hours at room temperature. After washing the wells, 100 µL Streptavidin-HRP Working Conjugate was added to each well and the plate incubated for 30 minutes at room temperature. After washing, 100 µL of Stabilized Chromogen were loaded in each well and the plate was incubated at room temperature in the dark until the appearance of the “blue color”. Finally, 100 µL of Stop Solution were added to each well and the plate was read with the Spectramax Plus spectrophotometer at the absorbance at 450 nm.

4.2 Results

4.3.1 Cord blood rescues stroke-induced spleen weight reduction, which correlates with brain infarct volumes.

In Figure 4.1A, it is clear that spleens from animals subject to stroke surgery were smaller than those from sham animals. When the spleens were weighed this decrease was significant (p<0.05) (Figure 4.1B). More importantly, HUCBC infusion reversed this weight loss and size reduction to values comparable to the non-stroked sham rats (Figure 4.1A and 4.1B). While the average spleen weight of a stroke rat was 0.53±0.049 grams, spleens from stroked rats treated with HUCBC had an average weight of 0.67±0.06 grams, which was significantly greater than the weight for MCAO only animals (p <0.05).

TTC staining of the brain revealed that HUCBC infusion also significantly mitigated the extent of ischemic damage seen after MCAO (Figure 4.2A). As shown in Figure 4.2B
Figure 4.1
Cord blood rescues spleen weight reduction induced by stroke.
A. Photo of spleens harvested from sham, MCAO, and MCAO rats that received HUCBC. B. Spleen weight for the three groups (*p<0.05).
Figure 4.2
Reduction of MCAO induced ischemic volume by HUCBC infusion. A. Coronal sections of brains stained with TTC. B. Percent of infarct volume in MCAO rats and MCAO rats that received HUCBC. (*p<0.05).
and the volume of lesion was significantly decreased after the infusion of HUCBC (p<0.05). Correlation analysis demonstrated an inverse relationship between spleen weights and brain infarct volumes (r = -0.638; p=0.01) (Figure 4.3).

4.3.2 Effect of cord blood on phenotype of ischemic rat derived splenocytes.

Once we determined that stroke surgery and the subsequent HUCBC therapy induced a change in the spleen weight which correlated with the infarct brain, we sought to further explore whether this phenomenon could be paralleled by other changes within the spleen. First of all, we investigated whether the splenocyte profile could be altered by the MCAO and the HUCBC therapy. Flow cytometry revealed that stroke altered the ratio of CD8+/CD4+ lymphocytes in the spleen, but HUCBC infusion restored the normal cellular profile of the spleens. Specifically, the MCAO surgery decreased the percentage of CD8 positive splenocytes present (Figure 4.4A). When HUCBC were infused, the percentage of CD8 positive lymphocytes was comparable to levels of rats that had not undergone stroke surgery (Figure 4.4A). The reduction in the percentage of CD8 lymphocytes was not accompanied by a significant change in CD4 positive splenocytes in either MCAO or MCAO+HUCBC rat spleens. As a consequence the CD8/CD4 ratio after stroke surgery was decreased and subsequently reverted to normal with HUCBC infusion (p<0.05) (Figure 4.4B).
Figure 4.3
Inverse correlation between spleen weight and percent of brain ischemic volume. Pearsons’ correlation analysis between % brain ischemic volume and spleen weight shows ($r=-0.638$, $p=0.01$). Linear regression analysis as represented by the best fit line equal (shown in diagram) reveals an $r$-square=0.47
Figure 4.4
Stroke-induced changes in CD8+ cell in spleen are reversed by
HUCBC treatment.  A) Representative flow plots of the CD4+ and
CD8+ cell composition of the spleen for the three experimental
groups: control (sham), MCAO and MCAO+HUCBC.  B) Ratio of
CD8+/CD4+ (*p<0.05).
4.3.3 Effect of cord blood on production of pro- and anti-inflammatory cytokines in the spleen.

Given the above findings, we wondered whether the function of the spleen could also be somehow altered by the brain ischemic insult, and if cord blood could play a role in this phenomenon. Our first approach was to examine the cytokine profile from spleen homogenates of stroke rats and HUCBC-treated stroke rats. RNase protection assay was employed for this first “screening”. As shown in Figure 4.5 the mRNA levels of TNFalpha, IL-1beta and IL-6 were not significantly altered by the stroke surgery or by the subsequent injection of HUCBC, although between the sham rats and HUCBC-treated MCAO rats the levels of TNFalpha were significantly decreased (p<0.05). IFN-gamma, IL-10 and IL-6 all showed a tendency to increase after stroke surgery, with an additional increase after HUCBC infusion (Figure 4.6). However, statistical analysis revealed no significance in differences of IL-2 values between any of the experimental groups (sham vs MCAO, MCAO vs MCAO+HUCBC, sham vs MCAO+HUCBC) (p>0.05) (Figure 4.6). On the contrary, in the analysis of both IL-10 and IFN-gamma differences between the sham group and the MCAO+HUCBC group, and between the MCAO group and the HUCBC-treated group were significant (p<0.05) (Figure 4.6).

4.3.4 Cord blood depresses the splenic γ cell proliferation in stroke rats, enhancing the production of IL-10 and blocking the release of IFN-γ.

To better investigate the effect of cord blood infusion on the function of splenocytes of rats subjected to stroke, we sought to explore the proliferative and molecular response of splenocytes to B and T stimulants. The MTT proliferation assay on cultured splenocytes
Figure 4.5
Density quantification of RNAse protection assay for spleen sample mRNA levels of: A) TNF-α (*p<0.05), B) IL-6, C) and IL-1β.
Figure 4.6
Density quantification of RNAse protection assay for spleen samples: A) IFN-γ (*p<0.05), B) IL-10 (*p<0.05) and C) IL-2.
harvested from sham, MCAO and HUCBC-treated MCAO rats, demonstrated that stroke increased the sensitivity of splenocytes to the T cell mitogen, Con-A (p<0.05) (see Figure 4.7). In contrast, infusion of cord blood cells 24 hours prior to harvesting the spleens of MCAO rats led to a significant reduction in T-cell proliferation in response to Con-A (p<0.05) (see Figure 4.7). B-cell proliferation, as tested with the stimulation of cultured splenocytes with LPS, was not significantly altered by stroke surgery or with subsequent injection of cord blood (see Figure 4.7).

Given the dramatic effect of cord blood infusion on the splenocyte response to a T cell mitogen (Con-A), supernatant from Con-A treated splenocyte cultures of all experimental conditions was collected and ELISA performed. Stroke did not significantly increase the production of IFNgamma from Con-A stimulated splenocytes as compared to cultures of sham rats, whereas IL-10 release was dramatically decreased (p<0.01) (see Figure 4.8A and 4.8B). In contrast, HUCBC treatment dramatically suppressed the expression of IFNgamma and considerably enhanced the production of IL-10 (p<0.01) compared to MCAO derived splenocytes (see Figure 4.8A and 4.8B).

4.4 Discussion

In our previous studies (see Chapter 3) (Willing, Lixian et al. 2003; Willing, Vendrame et al. 2003), we described a neuro-protective effect of HUCBC in the rat MCAO model of stroke. In this study, we have extended those finding by showing an earlier rescue from MCAO induced brain damage than previously reported. Moreover, we describe a direct effect of HUCBC on the spleen as demonstrated by the reversal of MCAO associated
Figure 4.7
Proliferation assay on splenocytes from sham, MCAO and MCAO+HUCBC rats. Y axis indicates the MTT Proliferative Index after stimulation with LPS (light grey bars) and Con-A (dark grey bars). *p<0.05.
Cytokine ELISA on supernatants of splenocytes cultures stimulated with Con-A. A) IFN-gamma secretion was significantly decreased in the MCAO+HUCBC group (*p<0.01).
B) IL-10 secretion was decreased in the MCAO group and augmented in the MCAO+HUCBC group (*p<0.01).
spleen size reduction; interestingly, among all the MCAO animals we found an inverse correlation between spleen weight and the extent of brain infarction.

The spleen shrinkage following stroke has been previously proposed to mirror a mobilization of lymphocytes from the spleen (Gendron, Teitelbaum et al. 2002).

Additionally, the CD8+ lymphocytes, which are T-lymphocytes with cytotoxic properties, have been shown to be significantly elevated in the ischemic hemisphere 48 hours following MCAO in the rat (Schroeter, Jander et al. 1994). In support of these observations, we also found decreased numbers of CD8+ lymphocytes in the spleens from MCAO rats; however, HUCBC treatment returned spleen CD8+ percentages to pre-stroke levels. These results suggest that the spleen could be a reservoir for the CD8+ T-lymphocytes that are mobilized following CNS injury and importantly that HUCBC protection in MCAO rat could be partly derived from the modulation of spleen CD8+ T-lymphocyte function.

Functional characterization of the splenocytes using the lymphocyte mitogen Con-A suggested that following MCAO, the spleen was sensitized (in terms of proliferative response) to such stimuli. Additionally the profile of cytokines elicited by Con-A treatment of splenocytes derived from the MCAO rats show elevated levels of IFN-γ and decreased IL-10 production. In contrast, when challenged with Con-A, splenocytes from MCAO rats that received HUCBC infusion induced increased IL-10 and decreased IFN-γ release compared to MCAO rats. This functional characteristic of the spleen cells indicates that following stroke injury the peripheral immune response (as governed by the spleen) may be skewed towards a Th1-type response, i.e. the pro-inflammatory reaction to the injury. HUCBC infusion changed this response to a Th2-type response (greater
ratio of CD8+/CD4+ splenocytes that release IL-10 while demonstrating only minimal proliferation). Such phenomenon may represent a mechanistic explanation for the beneficial effect induced by cord blood infusion as shown in our studies relating to stroke and by others in various models of brain injury. Interestingly, IL-10 has been reported to directly mediate infarct size reduction when systemically delivered into MCAO rats (Spera, Ellison et al. 1998), or when secreted endogenously by mobilized CD4+ T-lymphocytes (Frenkel, Huang et al. 2003). In addition to the increased protein levels of IL-10 in the culture supernatant of Con-A treated splenocytes derived HUCBC recipient MCAO rats, RNase protection assay demonstrated that mRNA levels of IL-10 from spleens of the same group were also significantly increased compared to control or MCAO rats.

HUCBC is well known for its low immunogenicity; nonetheless, given the lack of immunosuppressive therapy in the experiments (Chapter 4 and 5) examining HUCBC effects on MCAO associated immune alterations, it is possible that some of the outcome we described might be related to a graft-versus-host phenomena. However, the immunomodulatory effects we describe are consistent with reports showing that HUCBC can functionally repopulate the lymphoid organs (including spleen) in mice, which are genetically immunodeficient (Traggiai, Chicha, et al., 2004). We suggest that this immunomodulatory function of HUCBC may be comparable to that of adoptive transfer of splenocytes as therapy for MCAO and other diseases of brain injury. This phenomenon might represent a prototype mechanism by which HUCBC mediate the neurobehavioral recovery as described not only in stroke but also in other neuro-inflammatory/degenerative disease models.
CHAPTER FIVE

MODULATION OF STROKE-INDUCED BRAIN INFLAMMATORY EVENTS BY CORD BLOOD INFUSION

5.1 Introduction

Brain ischemia induces a cascade of inflammatory events which contributes to the extent of CNS damage. Resident macrophages such as microglia are activated and participate in the production of a number of substances including pro-inflammatory cytokines such as TNFalpha and other molecules with neurotoxic properties, like toxic free radicals (Liu, Clark et al. 1994; Buttini, Appel et al. 1996; Liu, Grossman et al. 2001). Concomitantly, a number of different types of circulating inflammatory cells infiltrate the infarcted brain tissue. Examples of those are lymphocytes, neutrophils and macrophages (Schroeter, Jander et al. 1994; Jander, Kraemer et al. 1995; Stevens, Bao et al. 2002). The literature regarding the timeline of extravasation from blood into brain of these different cell populations is quite heterogeneous. For example, using flow cytometry to identify macrophages in the mouse brain after ischemia Stevens et al. reported an increase in number of macrophages (either resident microglia or blood borne macrophages) starting at 18 hours after MCAO which peaked at four fold above that of control non-stroke animals at 48 hours after MCAO (Stevens, Bao et al. 2002). In another study, immunohistochemistry techniques have shown that there is an increase in infiltrating macrophages at 1 to 2 days after stroke which increases progressively over the following 5 days (Jander, Kraemer et al. 1995). Other studies have shown that this
increase can be prolonged up to 14 days after stroke, and macrophages can be identified within the ischemic tissue up to 30 days after the stroke induction (Schroeter, Jander et al. 1994). In the study by Stevens et al, T cells were shown to be significantly increased 48 hours after MCAO, to reach their peak of expression at the 72-hour time point, and remain elevated up to 4 days post-ischemia (Stevens, Bao et al. 2002). Similarly, immunohistochemical studies of Schoetler et al. (Schroeter, Jander et al. 1994) and Jander et al. (Jander, Kraemer et al. 1995) found T cell infiltration at 48 to 72 hours of stroke induction that continued to increase over the following 7 days. Contrary to T cells, the presence of B cells in the brain is not documented as significantly augmented post-ischemia (Stevens, Bao et al. 2002). Flow cytometry studies have shown that neutrophils infiltrate into stroked brain peaking at 48 hours post-ischemia (Stevens, Bao et al. 2002).

Inflammatory cell infiltration into the brain after ischemic insult has been studied quite extensively because controlling the infiltration of these cells could constitute a protective intervention in the ischemic brain. Indeed, the process of tissue loss after an ischemic insult is thought not to be completed for hours or days. As explained in the Introduction chapter, the so called “penumbra” is the area of brain infarct in which highly complex pathological processes induced by the ischemia are slowly progressing and as such are still reversible. With the idea that the progression of the injury in the penumbra can be affected by manipulation of the inflammatory response, agents that block the migration of cells into the brain have been employed in several studies. Antibodies that prevent leukocyte adhesion to endothelia, such as antibodies to the inter-cellular adhesion molecule-1 (ICAM-1), have been reported to decrease ischemic damage in animal models of stroke (Zhang, Chopp et al. 1994). Similarly, therapeutic interventions blocking the
CD11b molecule, the ligand of ICAM-1, have been demonstrated to reduce infarct volume and ameliorate functional deficits in the MCAO model of stroke (Chen, Chopp et al. 1994). However, as I also pointed out in the Chapter 1, the use of such agents in clinical trials has produced equivocal results. Despite this, in the research setting there are still hopes for the discovery of an effective blockade of the pro-inflammatory cell infiltration.

Taking into account our results illustrated in the previous chapters (Chapter 3 and Chapter 4), we sought to investigate whether HUCBC infusion could somehow have an effect on the cellular events that occur in the brain after ischemic injury. In particular, the fact that intravenously delivered HUCBC had systemic immune-regulatory effects by modulating the function of splenic cells of stroked rats, led us to investigate whether these cells could modulate the presence of immune/inflammatory cells within the ischemic brain.

To address this question, we used flow cytometry to characterize immune/inflammatory cells in the stroke brain because it offers a number of advantages over immunohistochemistry. First of all, it allows a quantitative analysis on the entire hemisphere, which is sensitive and fast. Secondly, the possibility of performing it exclusively on live cells, allows us to exclude possible artifacts due to non-specific antibody binding or background signals of interference. However, the disadvantage that for years has held investigators back from using it on tissue is that the single cell suspension is difficult to obtain and may harm the cells and cell surface markers. Recently several investigators have attempted to isolate different brain cell subpopulations for the flow cytometry analysis (Ford, Goodsall et al. 1995; Campanella,
Sciorati et al. 2002; Stevens, Bao et al. 2002). These techniques were modified in our laboratory to provide reliably reproducible cell suspensions from the ischemic brain.

Another approach that we adopted to examine the effect of cord blood infusion in the modulation of brain inflammatory events following stroke was based on the investigation of brain cytokine profiles. Brain cells including microglia, astrocytes, endothelial cells and neurons can produce cytokines. The most potent pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-alpha), is produced by microglial cells within the first 3 hours post-ischemic insult (Buttini, Appel et al. 1996). Although some studies have shown that this cytokine can possess neuroprotective properties, these are thought to be confined to regulation of calcium homeostasis in the neuron (Cheng, Christakos et al. 1994).

Generally, TNFalpha is known to be neurotoxic and its upregulation is associated with neurodegeneration and microgliosis (Sredni-Kenigsbuch 2002). Together with TNF-alpha, the interleukin-1 beta (IL-1beta) is considered another important player in the ischemic brain. Increased levels of this cytokine have been documented as soon as 2 hours post-MCAO (Zhai, Futrell et al. 1997) and studies on MCAO mice lacking its receptor (IL-1beta receptor knock-out mice) have shown a significant decrease in oxidative neuronal damage compared to wild type mice (Ohtaki, Takaki et al. 2003).

Blockage of pro-inflammatory cytokines, either by blocking their expression or abrogating their function, has been the focus of several therapeutic strategies in the past. For instance, overexpression of the IL-1 receptor antagonist induced with a viral vector has been shown to attenuate the inflammatory response that follows MCAO (Yang, Liu et
al. 1998). Curiously, the blockage of IL-1beta production is thought to be the mechanism by which electroacupuncture is an effective treatment after ischemic brain injury in China (Xu, Wu et al. 2002). Other therapeutic and experimental approaches have been based on the blockage of pro-inflammatory cytokines either by the use of antibodies neutralizing their activity, or by the inhibition of enzymes involved in their production. For example, inhibition of TNFalpha with antibodies administered intraventricularly to MCAO mice is effective in reducing the lesion volume (Yang, Gong et al. 1998). Similarly, pharmacological inhibition of the TNFalpha converting enzyme 30 minutes before the induction of the stroke, can minimize the infarct volume and promote functional benefits in the MCAO rats (Wang, Feuerstein et al. 2004).

To examine whether cord blood cells could have a role in the regulation of the expression of pro- and/or anti-inflammatory cytokines, we determined the messenger RNA and protein levels of an array of cytokines within the entire ischemic brain.

5.2 Materials and Methods

Adult male Sprague Dawley rats were randomly assigned to the following experimental groups: sham surgery (n = 16), MCAO only (n = 18) or MCAO plus HUCBC (n = 18). MCAO surgery was performed as previously described in Section 2.1. Rats received cord blood cells transplant 24 hours after the stroke surgery. An amount of 10,000,000 cells was injected as explained in detail (Chapter 2, paragraph 2.2.3). The following day, all rats were killed by decapitation after induction of anesthesia with isoflurane. Half of the animals underwent RNA analysis and half protein analysis with either cytokines array
or ELISAs. The rat brain was removed and the entire right hemisphere was rapidly dissected. We decided not to dissect specific brain areas but to analyze the right hemisphere, since permanent middle cerebral artery occlusion generally provokes a lesion that extends along the entire hemisphere, and cytokines rapidly diffuse within the microenvironment (Buttini, Appel et al. 1996). The brain hemispheres of animals for RNA analysis were immediately immersed in Trizol reagent in 1.5 ml RNAse-free Eppendorf tubes. RNA was isolated and RNAse protection assay was conducted as previously described for the spleen samples (for details, see Chapter 4, paragraph 4.3). The brains for protein analysis were stored in 1.5ml of lysis buffer at –80 ºC until usage. Brains were homogenized and cytokines levels were measured as explained in detail in Chapter 4 paragraph 4.3.

For the flow cytometry studies, another set of 12 animals was employed. These were assigned to the following experimental groups: sham surgery (n = 4), MCAO only (n = 4) or MCAO plus HUCBC (n = 4). Rats underwent stroke surgery and HUCBC transplant 24 hours after according to the methods described previously (Chapter 2, paragraph 2 and 3). The day after the transplant, rats were killed by decapitation, the brains were rapidly removed and freed from the meninges. Single cell suspensions were obtained with Percoll gradient separation as described below.

### 5.2.1 Separation of cells by Percoll gradient
The brain hemispheres were separated from the meninges and placed into 15 mL of ice-cold HBSS containing 0.2% bovine serum albumin (BSA) (Sigma), 0.01 mol/L EDTA, and 10 mg/mL deoxyribonuclease I (Sigma). Figure 5.1 represents a schema of the procedure. Brain tissue was homogenized using a Dounce homogenizer and passed through a 40-µm nylon cell strainer (Becton Dickinson). The suspension was centrifuged at 400g for 10 minutes at room temperature; the pellet was resuspended in 10 mL of HBSS containing 20U/mL collagenase II (Sigma) and centrifuged at 400g for 10 minutes at room temperature. Finally the pellet was resuspended in 2 mL of 70% Percoll (Gibco) in a 15 mL tube, which was overlaid with a gradient containing 2 mL of 30% of Percoll solution and 2 mL of PBS. Percoll was prepared by dilution in PBS. The gradient was centrifuged at 600g for 45 minutes at room temperature; cells were collected from the 30% layer to the PBS interface (about 5 mL) and washed with HBSS containing 10% fetal bovine serum (FBS) (Gibco).

5.2.2 Immunolabeling protocol and preparation of samples for flow cytometry analysis.

The following antibodies were used in the flow cytometry assay: monoclonal anti-rat CD11b fluorescein isothiocyanate (FITC)–conjugated antibody, monoclonal anti-rat CD45 phycoerythrin-conjugated antibody, monoclonal anti-rat CD3 antibody conjugated with phycoerythrin (PharMingen), monoclonal anti-rat B220 phycoerythrin-conjugated antibody, monoclonal anti-rat neutrophils antibody conjugated with phycoerythrin (PharMingen). Isotype controls (PharMingen) were used in parallel.
Cells were then incubated in the dark with the appropriate fluorochrome conjugated antibody for 60 minutes on ice. Afterwards, cells were washed three times with 500 µl of flow cytometry buffer (0.02% BSA, 0.05 mM EDTA in PBS).

5.2.3 Acquisition and analysis of data

The cells were resuspended in 200 µl of flow cytometry buffer containing Fc-block™ (BD Pharmingen) to reduce non-specific antibody binding. Acquisition was performed with the FACSCalibur (Becton Dickson) and CELLQuest software (Becton Dickinson). Dead cells were excluded from the analysis by ToPro-3 staining. Data were analyzed using CELLQuest software (Becton Dickinson).

5.3 Results

5.3.1 HUCBC infusion reduces the stroke-induced upregulation of CD45/CD11b positive and B220 positive cells within the brain

FACS revealed that CD45/CD11b positive cells were increased in stroke brains relative to brains from sham controls with an average of 5-fold increment (Figure 5.2 and 5.6). When HUCBC were delivered 24 hours after the stroke surgery, this population of CD11b/CD45 positive cells was dramatically diminished (Figure 5.2 and Figure 5.6). Statistical analysis conducted on the results of four distinct observations revealed that this reduction was significant (p<0.01).

For the analysis of B cells the B220/CD45R marker was used. Analysis showed a 2-fold increment in B220 positive cells in brains that had undergone MCAO (Figure 5.3 and
Figure 5.1
Schematic representation of the protocol for isolation of brain cells for flow cytometric analysis.
Figure 5.2
Flow cytometry detection of microglia/monocytes by gating of CD45/CD11b+ cells in Sham, MCAO and MCAO+HUCBC rat brains.
Figure 5.2
Flow cytometry detection of microglia/monocytes by gating of CD45/CD11b+ cells in Sham, MCAO and MCAO+HUCBC rat brains.
Figure 5.3
Flow cytometry detection of B-cells by gating of CD45/B220+ cells in Sham, MCAO, and MCAO+HUCBC rat brains.
Figure 5.4
Flow cytometry detection of T-cells by gating of CD45/CD3+ cells in Sham, MCAO, and MCAO+HUCBC rat brains.
Figure 5.5
Flow cytometry detection of neutrophils by gating of CD45/NT cells in Sham, MCAO, and MCAO+HUCBC rat brains.
Figure 5.6
Fold increase of inflammatory cells in brains of MCAO and HUCBC-treated animals over those obtained from sham rat brain. (* p < 0.01; # p < 0.05).
In brains of MCAO rats that received HUCBC infusion there were significantly fewer B220 cells compared to brains of MCAO-only rats (p<0.05) (Figure 5.3 and 5.6).

5.3.2 **HUCBC infusion does not alter the presence of CD45/CD3 positive cells within the ischemic brain.**

Lymphocyte populations were studied with the use of an antibody specific for the CD3 marker. After stroke surgery, the percentage of cells that were CD45 and CD3 positive was augmented by 2-fold (Figure 5.4 and 5.6). When HUCBC were injected, there was no significant alteration in the number of CD45/CD3 positive cells (Figure 5.4 and 5.6).

5.3.3 **The percentage of Neutrophils is not altered 48 hours after stroke surgery.**

Although the amount of neutrophils was increased in some brains from stroke rats, this was not a consistent observation. When the results were averaged across animals, there was no significant increment in the percentage of neutrophils present after MCAO (Figure 5.5 and 5.6). Additionally, after treatment with HUCBC this percentage was not further altered (Figure 5.5 and 5.6).

5.3.4 **Messenger RNA expression of pro-inflammatory cytokines is regulated by the injection of cord blood cells.**

Using the RNase protection assay we were able to analyze a wide array of cytokines including: TNFalpha, IL-1beta, TNFbeta, IL-2, IL-6, IL-10, IL-4, INF-gamma and TGF-beta. INF-gamma and TGF-beta were undetectable in any experimental conditions.
Figure 5.7
Density quantification of the RNAse protection assay in brain for the three indicated experimental groups. A) TNFalpha (*p<0.05) B) IL-1 beta (*p<0.05).
Figure 5.8
Density quantification of the RNase protection assay of brain for the three indicated experimental groups. A) TNFbeta (*p<0.05) B) IL-2 (*p<0.01 between sham and MCAO, *p<0.05 between MCAO and MCAO+HUCBC).
Figure 5.9
Density quantification of the mRNA levels of A) IL-6, B) IL-10 (*p<0.05), and C) IL-4 (*p<0.05) in the brain by using the RNAse protection assay on the three indicated experimental groups.
In brains of MCAO rats that received HUCBC infusion there were significantly fewer B220 cells compared to brains of MCAO-only rats (p<0.05) (Figure 5.3 and 5.6).

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Figure 5.10
Brain protein levels, under the indicated treatment groups, for A) TNFalpha and B) IL-1beta as determined by their respective cytokine specific ELISAs (all *p<0.05).
Figure 5.11
Brain levels of levels, under the indicated treatment groups, for (A) IL-2, (B) IL-6 (c) IL-10 as determined by ELISA (*p<0.05).
Similarly, mRNA levels of IL-4 were decreased in brains of rats subjected to stroke (p<0.05) (Figure 5.9), but the injection of HUCBC did not modify this effect, leaving IL-4 mRNA at levels comparable to MCAO only animals (Figure 5.9).

5.3.5 HUCBC infusion depresses the expression of pro-inflammatory cytokines that are upregulated within the ischemic brain.

Cytokines ELISAs were performed for TNFalpha, IL-1beta, IL-2, IL-6, IL-10 and interferon-gamma (IFNgamma). The IFNgamma was the only cytokine which was not detectable in any experimental conditions.

The expression of TNFalpha was significantly higher in the stroke animals (MCAO group) compared to the sham animals (p<0.05) (Figure 5.10A). When cord blood was injected, the levels of TNFalpha dropped to a level comparable to that of the normal sham rats. Interestingly, this cord blood-dependent drop was statistical significant (p<0.05) (Figure 5.10A).

When IL-1beta was measured, it was higher in the brains of the MCAO rats, relative to brains from sham rats (p<0.05) (Figure 5.10B). After injection of cord blood, there was a significant decrease of IL-1beta compared to the non-treated MCAO only rats (p<0.05) (Figure 5.10B).

Interleukin-2 was detected in higher amounts from ischemic brains in comparison to non-ischemic brains (p<0.05) (Figure 5.11A). While injection of cord blood appeared to reduce these amounts, this decrease was not statistically significant (p>0.05) (Figure 5.11A)
Similar to the results on the IL-6 mRNA, the detection of IL-6 revealed a considerable variability across the animals subject to stroke. The levels of IL-6 seemed higher in group of animals relative to the non-stroke animals, but this difference was not statistically significant (p>0.05) (Figure 5.11B). There was a reduction of IL-6 levels induced by the injection of cord blood, but also this reduction did not reach significance (Figure 5.11B). When IL-10 was measured, its levels were higher in the MCAO brain relative to sham brains, this difference was not significant (p>0.05) (Figure 5.11C). Levels of IL-10 in the brains of rats treated with cord blood cells were comparable to those of MCAO rats (p>0.05) (Figure 5.11C).

5.4 Discussion

These data suggest that in ischemic rat brain, CD11b/CD45 positive cells are increased in number, and infusion of HUCBC depresses this phenomenon. In particular the reduction in percentage of CD11b/CD45 positive cells appeared to be quite dramatic (from a 5-fold increase to baseline levels). Previous studies have suggested that this population of CD11b/CD45 positive cells, that is mainly constituted by the so called “CD11b/CD45 highly bright” cells (i.e. displaying brighter fluorescence for the CD45 marker) are actually microglial cells (Ford, Goodsall et al. 1995). Accepting this idea, the finding that HUCBC can reduce the number of microglial cells that are augmented by the stroke is a result is of particular interest, given that microgliosis is thought to take part in neuronal damage not only in consequence of ischemic injury, but also in other neurodegenerative diseases (Streit, Walter et al. 1999). Cord blood may block this
phenomenon, and this effect may be related to the beneficial neurobehavioral effects that cord blood is shown to promote. Future studies will be directed in investigating this phenomenon more closely.

B cells were detected in higher amount in the MCAO brains relatively to sham operated rat brains, a finding that is uncommon in the literature (Stevens, Bao et al. 2002). Moreover, the role that B cells could have in the pathogenesis of the ischemic brain injury is completely unknown. Although humoral responses (as B cell immune responses represent) are generally seen much later after an injury, an early humoral response could be evoked after a brain injury, and the presence of B cells within the brain parenchyma could represent the migration of these cells through the disrupted blood brain barrier (BBB). The subsequent infusion of HUCBC decreased the number of B cells to pre-stroke levels, but given the above observations the significance of this finding remains unclear. However, HUCBC might play a role in decreasing the humoral response seen after a brain insult, and this phenomenon could be reflected in a decrease of B cells in the brain.

When T cells were analyzed, although there was a 2-fold increment induced with stroke surgery, no additional variations were observed when cord blood was infused. In some animals the percentage of T cells was increased with MCAO surgery, but this phenomenon was not observed for all animals. T cells have been shown to possess neuroprotective properties in the ischemic brain (Wolf, Fisher et al. 2002), but whether cord blood cell infusion may modulate their expression within the stroke brain is not clear. Finally, the neutrophilic populations were not found significantly altered by either
stroke surgery or the subsequent injection of cells. Further research may be directed to the analysis of the same events at further time points.

To summarize of the flow cytometry studies, the inhibition of stroke-induced up-regulation of CD45/CD11b positive cells by infusion of cord blood may be of interest as a putative mechanism of action of the HUCBC. This result holds particular significance given that such a “indirect” mechanism of action of the HUCBC is supported by the previously presented immunohistochemical findings of scarce cells within the brain parenchyma or within the cerebrovasculature.

The investigations of cytokine production in the brains of stroke rats and of stroke rats that received the HUCBC treatment also provided interesting results. Over-expression of TNFalpha, both at the level of RNA synthesis and protein production, was induced by the ischemic insult. Injection of cord blood significantly depressed these phenomena, inducing a decrease in RNA production and a concomitant decrease also in protein production. These findings are of considerable interest, given the role that is attributable to TNFalpha in neuronal degeneration (Sredni-Kenigsbuch 2002). Cord blood may exert its neuroprotective properties in the ischemic brain by modulating the expression of TNFalpha. In particular, this finding is consistent with the previous finding from the flow cytometry analysis suggesting that that HUCBC might have an inhibitory action on the stroke-induced microgliosis.
The mRNA level of TNFbeta was also found to be significantly decreased after HUCBC treatment. TNFbeta (also termed lymphotoxin) is a cytokine that has also been described as an important inflammatory mediator similar to TNFalpha (Ermert, Pantazis et al. 2003). Although there is little literature on the role of TNFbeta in the pathogenesis of stroke, its depression by HUCBC treated MCAO rats is in accordance with the effects on TNFalpha expression.

The IL-2 mRNA and protein levels were significantly upregulated in the CNS after the ischemic insult. However, the decreased IL-2 mRNA expression after the cord blood infusion (as seen by RNAse protection assay), was not mirrored by a similar decrease in its protein levels. This mismatch can be related to differences in timing between mRNA transcription and protein translation, or may simply reflect circulating levels of IL-2 protein from the periphery. It is important to remember that after an ischemic insult, brain and peripheral systems engage in a complex inter-play mediated by cytokines, chemokines and neurotransmitters (Woiciechowsky, Schoning et al. 1999). IL-2 may be involved in such a phenomenon, either manifesting its pro-inflammatory properties or simply orchestrating the dialogue between brain cells and immune peripheral actors during the post-ischemic systemic inflammatory reaction.

While there was a tendency in both protein levels and mRNA levels of IL-6 to be upregulated in the stroke brains; analysis revealed that this effect did not reach significance.

Anti-inflammatory cytokines were also examined. Messenger levels of IL-10 and IL-4 were significantly reduced after stroke surgery. Very likely this dramatic decrease represents the depression of the anti-inflammatory reaction that takes place concomitantly
to the activation of the pro-inflammatory response. Interestingly, protein levels of IL-10 were higher in stroke animals compared to sham animals. While this effect did not reach statistical significance, it may reflect a similar phenomenon to that observed with the mismatch in IL-2 mRNA and protein levels. It is well documented that after a brain ischemic event IL-10 is rapidly produced by peripheral cells (Woiciechowsky, Schoning et al. 1999), and this circulating IL-10 reaches the brain. Given this, the high levels of IL-10 we detected within the brain may be actually IL-10 produced in the periphery; supporting this idea we found that IL-10 mRNA levels in the brain did not parallel those IL-10 protein expression. After the injection of HUCBC, mRNA levels of both IL-10 and IL-4 were increased compared to MCAO rat brains, although not significantly. HUCBC transplantation may be promoting the anti-inflammatory response in the brain, while in parallel inducing a depression of the pro-inflammatory phenomena. Taken together, these observations suggest that following the HUCBC intravenous transplant, there is a blockage of the brain pro-inflammatory events induced by the ischemic insult. This blockage may result in histological and further behavioral benefits that we and others have observed.
CHAPTER SIX

CONCLUDING REMARKS

The first time cord blood cells were used in animal models of neurodegenerative diseases took place four years ago, when intravenously injected HUCBC were shown to prolong life span in models of Parkinson’s, Alzheimer’s and Huntington’s disease (Ende and Chen 2001; Ende, Chen et al. 2001; Ende and Chen 2002). While these preliminary studies showed an overall non-specific effect of HUCBC across these models, further research demonstrated that intravenously delivered cord blood cells were able to specifically ameliorate neurobehavioral deficits associated with brain ischemia (Chen, Sanberg et al. 2001; Willing, Lixian et al. 2003). The first report in a rodent stroke model showed that 1,000,000 cells transplanted intravenously 24 hours after the induction of the stroke, induced a considerable reduction of the stroke-induced impairments in motor-coordination tests (such as the rota-rod test) and ameliorated the motor-sensory parameters evaluated with the mNSS scale (Chen, Sanberg et al. 2001). The same investigators showed that injected cells were detected within the brain parenchyma as far as 14 days after the transplant. Furthermore migration studies supported the hypothesis that these cells possess tropic properties and migrated to the injured brain (Chen, Sanberg et al. 2001). The intriguing question that remained after this study was whether these cells were capable of inducing these effects by differentiating into neural or glial cell types, or
whether their effect was related to other mechanisms outside neural differentiation. Subsequent studies began to point to other mechanisms. It was surprising to discover that whether HUCBC were delivered directly into the corpus striatum or infused via the femoral vein the neurobehavioral benefit assessed with tests of spontaneous activity or motor asymmetry was equal (Willing, Lixian et al. 2003). The investigators pointed out that in some tests the improved motor function induced by intravenous delivery was even better than that induced with intracerebral transplant (Willing, Lixian et al. 2003). However, the cells were not detected within the brain parenchyma following either of the two transplant modalities, leading the investigators to hypothesize that HUCBC could exert their beneficial action though indirect mechanisms on the brain.

6.1 Neurobehavioral and Neuropathological benefits induced by HUCBC infusion in the MCAO animal model of stroke

The first important finding of the studies reported herein is that HUCBC can control the neurobehavioral deficits induced by brain ischemia in a manner that is dependent on the number of cells delivered. At least 1 million cells are necessary to achieve a stable rescue of deficits. When 10 million cells are employed, an even better effect can be achieved. However, particularly the spontaneous activity measurement revealed that higher cells doses, such as cell amounts in the range of 30 to 50 million do not induce a further recovery. Not only were there significant improvements in behavior, but cord blood injection also induced histopathological recovery in the ischemic brain; this had not been reported before. Moreover, when 10 million HUCBC were infused 24 hours after the
stroke surgery, the lesion volume was significantly reduced as compared to the non-treated stroked animals; whereas, animals treated with less than 1 million cells while showing some reduction in damage (as evidenced by cytoarchitecture sparing and less presence of lacunar spaces). These ameliorations however, did not translate into a significant reduction in volume of damage.

Given all the HUCBC doses employed, only the two highest doses of HUCBC were found to induce both behavioral benefit and infarct volume reduction. One important observation regards the mismatch between the behavioral benefit and infarct volume reduction when doses of 1 million cells are used. While this dose can induce a significant benefit in terms of spontaneous activity measurements and motor abilities, the same dose cannot induce a reduction of lesion volume. One explanation is that at this dose HUCBC there may not be sufficient “effector” cells to provide protection to significantly ameliorate ischemic damage. As also pointed in Chapter 1, these results demonstrates the importance of using a battery of tests to examine functional outcomes in these animal models of ischemia. Indeed, the MCAO model is characterized by an intrinsic variability (i.e. lesions can have different distribution due to variability of the microvascular structures across the animals) and by the use of multiple measures of functional outcomes, deficits related to lesions in different locations are picked up by different behavioral tests.
6.2 What is the mechanism by which HUCBC exert their beneficial effects?

These findings led us to investigate the mechanisms through which HUCBC may induce functional and structural ameliorations in the MCAO rat model of ischemic brain injury. The first approach was to examine where intravenously injected cells were located within the body at one month after the transplant. The main question was whether HUCBC could target a specific organ and migrate to it, or whether they are dispersed (via the vasculature) non-specifically throughout the body. One month after the transplant cells were mainly located within the injured side of the brain, and in the spleen. Both these findings are of relevance. First, the fact that HUCBC may reach the injured brain supports previous theories of tropic properties if HUCBC for the injured tissue (Chen, Sanberg et al. 2001). HUCBC may be attracted to the site of the lesion by chemokines and specifically exert their action in situ. Within the brain, the HUCBC seemed to be mainly localized at the level of the cerebrovasculature. This finding may indicate that HUCBC can act without becoming integrated in the parenchyma, an idea in direct contrast to the original hypothesis HUCBC performed cell replacement. Results from the first work employing HUCBC in a stroke model (Chen, Sanberg et al. 2001) supported the idea that HUCBC may integrate and substitute for lost or dying neurons within the brain parenchyma. Intravenously transplanted HUCBC were found in the CNS and immunohistochemical analysis shows the expression of neural markers and glial markers (such as MAP-2 and GFAP respectively) by the transplanted cells (Chen, Sanberg et al. 2001). Support for functional differentiation of HUCBC into CNS cell types has been suggested by the work of investigators in other animal models of brain injury (Lu, Sanberg et al. 2002). Although these initial observations seem in contrast with our
findings, the expression of neural markers does not necessarily mean that HUCBC are undergoing a process of transdifferentiation, and therefore does not demonstrate that these cells once in situ can restore brain function by becoming newborn neural or glial cells.

The other target organ was the spleen. Here also the HUCBC seemed to be found within or near the vasculature. The presence of transplanted cells in the spleen could be evidence of the spleen removing dead or foreign cells out of the vasculature (as is the case for spent red blood cells). On the other hand, since cord blood is composed mainly of immature blood cells (T lymphocytes and monocytes), the spleen may represent an actual homing site for the HUCBC. This idea is in agreement with previous findings of investigators that have employed HUCBC in the amyotrophic lateral sclerosis mouse model (Garbuzova-Davis, Willing et al. 2003). In this study numerous cells were found in the spleen, and investigators hypothesized that the spleen may be the main target of intravenously injected cord blood cells.

Finding HUCBC in the spleen lends credence to the hypothesis that the immune/inflammatory response that follows a brain ischemic insult may be modulated by our cell therapy. This idea, as outlined in detail in the previous chapters, was that there might be a peripheral and central effect induced by cord blood that could explain the functional benefit, seen in this and previous studies. When we examined the effect of stroke and subsequent HUCBC delivery on the rat spleen, we obtained very striking results. Cord blood infusion rescued the spleen weight loss that occurred in response to the stroke injury, and concomitantly re-instated the normal CD4/CD8 phenotype profile that had been altered following MCAO. Perhaps more importantly, the reduction of
ischemic volume correlated inversely with the spleen weight, indicating that the effect of HUCBC on the spleen may be of pathogenic relevance for the rescue of the brain histological lesion. Furthermore, splenic T cell function changed after stroke and after transplantation, leading to decreased production of IFN-gamma and inducing expression of IL-10. Such results can be interpreted in the frame of past studies that have shown that T cells can have a neuroprotective effect (Wolf, Fisher et al. 2002; Becker, Kindrick et al. 2003). One hypothesis may be that HUCBC induce the endogenous T cells to promote survival of neural cells, but this hypothesis as now remains to be tested.

Another approach that we used to investigate the possible role of HUCBC in the modulation of the endogenous post-stroke immune/inflammatory alterations was to examine whether HUCBC influenced the cellular and molecular phenomena within the brain after stroke. HUCBC blocked the acute infiltration of inflammatory cells such as microglia and B cells into the stroke brain. At the same time, mRNA and protein levels of TNF-alpha were abrogated by the infusion of cord blood cells, while protein or RNA amounts of IL-1beta or IL-2 were also decreased. These results strongly suggest that HUCBC modulate endogenous events that occur in the brain after ischemia. Additionally, the levels of anti-inflammatory cytokines tended to be higher after HUCBC treatment. Although this effect did not reach significance, this phenomenon could represent the effect of cord blood on the inhibition of pro-inflammatory cytokines (such as TNFalpha, IL-1beta and IL-2) and the simultaneous induction of expression of anti-inflammatory molecules. These molecular phenomena are quite transient and therefore generally difficult to observe at a single time point. Future studies should be directed in following these events across time.
Taking all these finding together a possible mechanism by which cord blood cells have been repeatedly reported to promote functional recovery from ischemic injury may be uncovered. Firstly, HUCBC modulates the stroke-induced inflammatory response by blocking the production of pro-inflammatory cytokines and by reducing the over-expression of pro-inflammatory cells within the ischemic brain. Secondly, HUCBC have a peripheral effect on the spleen by reverting the alterations on splenocytes phenotype and function that are induced by stroke. The intracellular events mediating these phenomena remain unknown, but future studies will be directed to the understanding of the molecular pathways that are activated/suppressed during these events. For instance, the transcription factor NFkB, that is involved in the activation of pro-inflammatory cytokines such as TNFalpha, will be subject to analysis.

6.3 Conclusion

Cord blood has recently emerged as a therapeutic option for neurodegenerative diseases including stroke. As a result of its easy availability, its low immunogenic properties and the low risk of viral transmission associated with its use, it represents an attractive tool for future therapies. In addition, the intravenous delivery of these cells makes this therapeutic approach easily accessible to many patients.

Despite the successes in demonstrating the benefit of cord blood cells when applied into stroke models, the mechanisms underlying its effect had not been characterized prior to the studies described in the dissertation. This work has provided extensive progress in addressing this big question, forming a foundation for further research.
Before applying this cord blood based therapy in the clinic, further issues will need to be addressed. Here we addressed the dose of cells necessary to obtain an optimal benefit. Our best dose (10,000,000 cells) translates into almost 20 cords for a transplant into an organism of the human size. *In vitro* expansion techniques may serve as a tool to overcome this issue. The time-window still remains to be addressed, although all this work has been conducted by transplanting HUCBC 24 hours the stroke, as preliminary studies conducted in our laboratory had shown that this might be the optimal time to delivery. Finally, it is very important to take into account that although experimental results are very promising, the effectiveness of these transplants in humans are unknown. The efficacy of the transplant will be obviously influenced by factors such as the severity, stage and site of the pathology of the neurodegenerative disorder affecting the recipient. For instance, the location and the extent of the neuronal damage represent factors that need to be assessed before the transplant. Importantly, there will be the need to establish criteria based on clinical and pathological parameters in order to screen which patients will be good for transplantation.
REFERENCES


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Martina Vendrame graduated from the University of Padova School of Medicine in Padova, Italy in 1999. After which, she completed a post lauream medical training at the University of Groningen in the Netherlands. In the course of her PhD studies at the University of South Florida, she has contributed to many publications; including a first author manuscript in the prestigious journal *Stroke*. Martina is now (2004) a Neurology Resident at Temple University Hospital; Philadelphia, PA.