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Systemic Neutrophil Activation in a Mouse Model of Ischemic Stroke and Reperfusion

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Abstract

As a natural response to injury and disease, neutrophils activate, adhere to the microvasculature, migrate into brain tissue, and release toxic substances such as reactive oxygen species and proteases. This neutrophil response occurs when blood flow is returned to brain tissue (reperfusion) after ischemic stroke. Thus, the presence of activated systemic neutrophils increases the potential for tissue injury during reperfusion after ischemic stroke. Although experiments in rat models suggest that activated neutrophils play a pivotal role in cerebral ischemia reperfusion injury, little is known about systemic neutrophil activation during reperfusion following ischemic stroke in a mouse model. The purpose of this study was to characterize systemic leukocyte responses and neutrophil CD11b expression 15-min and 24-hr post-reperfusion in a mouse model of ischemic stroke. The intraluminal filament method of transient middle cerebral artery occlusion (tMCAO) with reperfusion or a sham procedure was performed in male C57Bl/6 mice. Automated leukocyte counts and manual white blood cell (WBC) differential counts were measured. Flow cytometry was used to assess systemic neutrophil surface CD11b expression. The data suggest that the damaging potential of systemic neutrophil activation begins as early as 15 min and remains evident at 24 hr after the initiation of reperfusion. In addition, because transgenic mouse models, bred on a C57Bl/6 background, are increasingly used to elucidate single mechanisms of reperfusion injury after ischemic stroke, findings from this study are foundational for future investigations examining the damaging potential of neutrophil responses post-reperfusion after ischemic stroke in genetically altered mouse models within this background strain.

Keywords

murine; flow cytometry; ischemic stroke; reperfusion; neutrophil

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States (Lloyd-Jones et al., 2009). The American Stroke Association estimated that 795,000 Americans would have a stroke in 2009, 89% of them being ischemic strokes. Cerebral ischemic stroke is defined as a lack of, or reduced, oxygen and blood flow to the brain. The timely return of cerebral blood flow, termed reperfusion, is the goal of clinical treatment for ischemic stroke. Contradictorily, reperfusion returns oxygen to tissues

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undergoing anabolic respiration, augmenting oxidative stress. In addition, reperfusion resumes the circulation of activated blood cells, specifically neutrophils. Neutrophil adhesion to activated vascular endothelium in the cerebral microcirculation occurs via neutrophil CD11b and endothelial ICAM-1 interactions. As activated neutrophils adhere to vessel walls and enter brain tissue, they release toxic mediators such as reactive oxygen species and proteases. Activated neutrophils also release cytokines that recruit additional inflammatory responders to injured brain tissue. During reperfusion, neutrophil adhesion also contributes to a no-reflow phenomenon caused, in part, by cell trapping in the microvasculature (Reynolds & McDonagh, 1989; Ritter & McDonagh, 1997; Ritter, Orozco, Coull, McDonagh, & Rosenblum, 2000). Localized cell trapping further decreases blood flow following reperfusion and increases the likelihood of neutrophil rolling, adhesion, and diapedesis into brain tissue (Sandoval & Witt, 2008). The sum of these responses extends ischemic injury; this additional injury is known as ischemia–reperfusion (IR) injury.

A majority of studies that use an animal model to investigate neutrophil-mediated IR injury focus on neutrophil accumulation and the presence of deleterious neutrophil products (reactive oxygen species and proteases) within brain tissue after ischemic stroke and reperfusion (Akita, Nakase, Kaido, Kanemoto, & Sakaki, 2003; De Simoni et al., 2004; Storini et al., 2005). In contrast, our laboratory, using whole-blood (WB) flow cytometry and intravital microscopy methods, investigates neutrophil-mediated intravascular events that precede neutrophil accumulation within tissue (Ritter et al., 2000; Ritter, Stempel, Coull, & McDonagh, 2005). Using a rat model of cerebral IR injury, we have observed significant neutrophil rolling and adherence to the cerebral microcirculation during early reperfusion after ischemic stroke and demonstrated an increase in neutrophil activation after 24 hr of IR.

Transgenic and knockout mouse models are being increasingly used to elucidate mechanisms contributing to pathophysiologic states. Genetically altered mouse models have been used extensively in the investigation of inflammatory mechanisms of cerebral IR injury (Connolly et al., 1996; De Simoni et al., 2004; Ducruet et al., 2008; Mocco et al., 2006). The C57Bl/6 mouse serves as the background strain for many of these models. However, systemic leukocyte responses and specific assessment of neutrophil surface expression of CD11b in a reliable and reproducible model of cerebral IR in the C57Bl/6 mouse strain have not been reported. Therefore, the purpose of this study was to characterize systemic leukocyte responses and neutrophil CD11b expression at 15-min and 24-hr post-reperfusion, using WB flow cytometry in a C57Bl/6 mouse model of transient ischemic stroke.

Materials and Method

Animals and Experimental Protocol

All experiments were performed in male C57Bl/6 mice (20–25 g; Jackson Laboratories, Bar Harbor, Maine, USA), housed at constant temperature ($21 \pm 1^\circ\text{C}$) and with 12-hr light/dark cycles (6 a.m. to 6 p.m.). Food and water were available ad libitum. All animal handling, surgical, and euthanasia procedures were conducted in compliance with the guidelines of the University of Arizona Institutional Animal Care and Use Committee, which oversees conformity with national laws (Animal Welfare Act) and accreditation policies (Association for Assessment and Accreditation of Laboratory Animal Care). Animals were transported to approved laboratory housing 12–24 hr prior to surgery to reduce stress responses on the day of surgery. Animals recovered from survival surgeries in a temperature controlled ($21 \pm 1^\circ\text{C}$), quiet room with 12-hr light/dark cycle and free access to food and water. Blood samples for flow cytometry were collected at either 15 min (R15) or 24 hr (R24) of reperfusion. All brain infarct data were collected at 24 hr of reperfusion. Figure 1 summarizes the experiment protocol used in this study.

Middle Cerebral Artery Occlusion and Reperfusion Surgery

A total of 31 animals were randomly assigned to groups. Transient focal ischemic stroke with reperfusion (transient middle cerebral artery occlusion; tMCAO) was delivered to 18 mice using the intraluminal filament method as previously described (Sampei et al., 2000; W. Zhang et al., 2008), whereas 13 animals underwent a sham tMCAO surgery comprised of Doppler probe placement, common carotid artery and external common carotid artery (ECA) vessel isolation, vessel cauterization, and cutting of ECA but excluding filament placement. Throughout all surgical procedures (tMCAO or sham), body temperature was monitored via rectal probe and continuously maintained between 35.5°C and 36.5°C with heating pad and heating lamp.

Mice were anesthetized initially with 5% isoflurane in a nitrous oxide/oxygen mixture (0.1 L/min and 0.4 L/min, respectively) for all surgical procedures. Anesthesia was maintained at 1–2% isoflurane throughout the surgical procedure. A small incision was made over the right temporalis muscle. The muscle was dissected to create a small tunnel to the area of the skull overlying the middle cerebral artery. A laser Doppler probe (Perimed) was positioned on the skull for continuous relative cerebral blood flow (rCBF) measurement. The Doppler probe and animal were secured to a surgical stage to minimize artificial changes to rCBF measures. Right common and external carotid arteries were first isolated after which the ECA was cauterized and cut. Using the ECA stub, a filament was introduced into the vasculature and advanced to the internal common carotid artery (ICA). All filaments were made prior to surgery using 6-0 nylon (Ethilon, Ethicon), blunted and silicone coated (Xantopren comfort light, Heraeus, New York, USA) to a final size of 0.21–0.25 mm. When the filament was visualized in the ICA, the common carotid artery was tied and the filament was then advanced past the pterygopalatine artery and to the ostia of the middle cerebral artery. Correct placement of the filament was verified by a drastic and sudden reduction in rCBF measured by Doppler probe.

To model severe ischemic stroke, the filament remained in place for 60 min; rCBF was continuously monitored throughout ischemia and averaged measures were recorded at 15-min intervals. After the ischemic period, the filament was withdrawn and the common carotid was untied, initiating reperfusion. The ischemic period was defined as a reduction in rCBF by at least 70% of baseline. Reperfusion was defined as a return of rCBF to at least 70% of baseline. Doppler rCBF was measured continuously for 15 min after filament removal. For animals to be included in data analysis, 60 uninterrupted minutes of ischemia followed by at least 15 continuous minutes of reperfusion were required.

Quantification of Infarct, Edema, and Petechial Hemorrhage

After 24 hr of reperfusion, 10 mice from the tMCAO group were euthanized. The brain was rapidly removed, placed in a brain slicer (Braintree Scientific Inc., Braintree, Massachusetts, USA), sliced into five 2-mm slices, and stained with a 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 20 min at 37°C; tissue viable at the time of slicing stains red while necrotic tissue remains white. Slices were stored in 10% formalin and scanned (HP Scanjet 4850) the following day to TIFF image files at 600 dots per inch. Color digital images of slices were assessed in a blinded fashion using Image J software (NIH) for infarct volume, edema, and petechial hemorrhage area. To quantify infarct, necrotic (white) and healthy (red) tissue in the ipsilateral (affected) hemisphere was quantified in the total hemisphere, cortex, and striatum. Corresponding healthy tissue was also measured in the contralateral (nonaffected) hemisphere. Anatomical areas were identified using a mouse brain stereotaxic atlas reference (Paxinos & Franklin, 2004). Total infarct volume and infarct volume specific to the cortex and striatum are reported as a percentage of the contralateral hemisphere (Funk et al., 2003; Ruehl et al., 2002). Petechial hemorrhage was

identified as clustered small red spots within the necrotic tissue (Nagaraja, Keenan, Fenstermacher, & Knight, 2008; R. L. Zhang et al., 2008). Using Image J software, the area of petechial hemorrhage was measured in each slice and is reported as a total sum of the areas (μm) present for each brain. An indirect measure of edema was calculated for the ipsilateral hemisphere as previously described: $\text{Edema (\%)} = [(\text{Ipsilateral hemispheric volume} - \text{contralateral hemispheric volume}) / \text{contralateral hemispheric volume}] \times 100$ (Ruehl et al., 2002).

Blood Cell Count

Citrated WB (120 μl) was set aside for an automated blood cell count (Beckman Coulter, maker). Samples were counted in duplicate. Blood smears were made in duplicate, fixed, and stained for manual white blood cell (WBC) differential counts. In a blinded fashion with respect to group, 100 cells per slide were counted using a light microscope (40 $\times$, Nikon Eclipse, E600). Lymphocytes, neutrophils, and monocytes were identified, counted, and recorded as percentages.

Flow Cytometry

WB processing—After 15 min or 24 hr of reperfusion (R15 or R24, respectively), blood was successfully collected from animals as previously described by our laboratory (Maes, Davidson, McDonagh, & Ritter, 2007). Briefly, animals were deeply anesthetized with 3–5% isoflurane in a nitrous oxide/oxygen gas mixture for 3–5 min. After a midline dorsal incision and visualization of vasculature, blood was drawn from the ascending vena cava using a 23-gauge needle into a 1 ml syringe prepared for a 1:10 citrate/blood dilution (Sigma). Mice were euthanized at each discrete time point (R15 or R24) after blood collection. WB was aliquoted into Eppendorf tubes for neutrophil CD11b labeling. First, WB was diluted 1:1 with filtered warm (37°C) Pharmingen Staining Buffer (SB, BD Biosciences) for a final volume of 100 μl WB-SB. Neutrophil activation positive control samples were incubated first with 5 μl lipopolysaccharide (LPS, Sigma #2680, diluted in PBS, final concentration 10 $\mu\text{g}/0.1\text{ ml}$) for 30min. To identify all granulocytes, samples were incubated for 25 min with a pan-granulocyte marker Phycoerythrin-Cy5-conjugated anti CD45 (BD Biosciences, catalog # 559135, 4.0 $\mu\text{g}/\text{ml}$). To specifically assess neutrophil activation via cell surface expression of CD11b, samples were incubated for 25 min with either fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b monoclonal antibody (BD Pharmingen, catalog #557396, 5 $\mu\text{g}/\text{ml}$) or FITC-conjugated rat IgG2b, κ monoclonal immunoglobulin isotype control (BD Pharmingen, catalog #553988, 5 $\mu\text{g}/\text{ml}$) simultaneous with CD45 antibody incubation. After LPS incubation, positive control samples were also incubated with CD45 and CD11b antibodies as described. All incubations were performed in a dark water bath at 37°C. Ice-cold filtered 1% paraformaldehyde was added to each sample to stop incubation and fix cells.

Flow cytometer acquisition and analysis—All data were acquired via FACSCaliber flow cytometer (Becton Dickinson); CellQuest Pro software was used to analyze FACSCaliber data. Daily calibrations were performed and identical instrument settings were maintained throughout the study. Neutrophils were identified by characteristic forward and side scatter patterns previously established (Maes et al., 2007; Ritter et al., 2005) and a gate was set around this population. This gated region was consistent throughout data acquisition and analysis. Cell fluorescence was measured on a log scale and reported as a geometric mean for CD11b in the CD45 positive and gated neutrophil population. Neutrophil surface expression of CD11b is reported as total fluorescence intensity (TFI). TFI accounts for both the percentage of cells gated and the fluorescence intensity of the identified cells within a sample and is calculated as $(\text{percentage cells gated}/100) \times \text{geometric mean}$ (La Bonte, Davis-Gorman, Stahl, & McDonagh, 2008; Ritter et al., 2005).

Statistical Analysis

Results are expressed as mean $\pm$ *SEM*. All data were assessed for normalcy and student *t*-test comparisons were used to determine statistical differences between groups; bivariate correlation testing was performed using SPSS software (16.0).

Results

Physiologic and Surgical Variables

The mortality rate at 24 hr for the sham and tMCAO groups were 0% and 6%, respectively. Of the tMCAO groups (R15 and R24), 88% of the animals met the defined inclusion criteria and were included in the study. Physiologic and surgical variables for the sham and tMCAO procedures are summarized in Table 1. There was no significant difference in surgical temperature. The surgical time in the sham group was significantly shorter than in the tMCAO group but the sham group required slightly more isoflurane throughout the procedure. The sham surgery included vessel isolation but excluded filament placement. Filament placement typically required an additional 15 min after vessel preparation and accounts for the extended surgical time reported in the tMCAO group when compared to sham.

Infarct Volume, Brain Edema, and Petechial Hemorrhage

In C57Bl/6 mice subjected to 60 min of ischemia and 24 hr of reperfusion ($n = 10$), 53.6% $\pm$ 4.3% of the cortex, 85.4% $\pm$ 9.6% of the striatum, and 26.2% $\pm$ 3.2% of the total hemisphere were necrotic (Figure 2). Infarct area (mm²) at 2, 4, 6, and 8 mm from frontal pole was 21.8 $\pm$ 5.6, 42.5 $\pm$ 3.0, 33.9 $\pm$ 3.1, and 19.0 $\pm$ 4.4, respectively (Figure 3). Of note, infarct area (mm²) at 6 mm from the frontal pole was significantly correlated with total infarct volume (mm³), $r = 0.81$, $p = .004$ (Figure 3 inset). Petechial hemorrhage was present in 7 of the 10 animals; in these animals, total area of petechial hemorrhage was 885 $\pm$ 314 μ m². Figure 4 is a representative image of infarct area and petechial hemorrhage (red spots located between brackets) within the infarct area. Calculated brain edema in the tMCAO group ($n = 10$) was 7.5% $\pm$ 1.4%. Neither edema nor petechial hemorrhage correlated with total infarct volume ($r = .36$ and $r = .41$, respectively). No injury was observed in the contralateral hemisphere.

Systemic Leukocyte Response

Total WBC count and percent lymphocytes, neutrophils, and monocytes for sham and tMCAO groups at 15 min and 24 hr of reperfusion are summarized in Table 2. After 24 hr, but not at 15 min, of reperfusion, total WBC count was significantly reduced ($p = .001$ and $p = .61$, respectively) compared to their respective sham groups. After 24 hr and 15 min of reperfusion, the percentage of systemic neutrophils was significantly increased ($p = .019$ and $p = .03$, respectively) compared to the sham groups.

Systemic Neutrophil CD11b Expression

Systemic WB was assessed for neutrophil surface expression of CD11b in four groups of animals: animals that underwent (a) sham surgery and 15 min of reperfusion ($n = 6$), (b) tMCAO surgery and 15 min of reperfusion ($n = 6$), (c) sham surgery and 24 hr of reperfusion ($n = 7$), and (d) tMCAO surgery and 24 hr of reperfusion ($n = 10$). When compared to their respective sham groups, there was a significant increase in neutrophil CD11b expression at both 15 min and 24 hr of reperfusion ($p = .012$ and $p = .003$; Figure 5A and B). At 15 min of reperfusion ($n = 6$), there was no significant increase in neutrophil CD11b expression after LPS stimulation when compared to sham ($n = 6$, $p = .057$); however, there was a significant increase in neutrophil CD11b expression after LPS

stimulation at 24 hr of reperfusion ($n = 9$) compared to the sham group ($n = 7$, $p = .001$; Figure 6a and b).

Discussion

Ischemic stroke occurs when a cerebral blood vessel is blocked by a thrombus or emboli resulting in complete cessation of blood flow or hypoperfusion to brain tissue. Cerebral ischemia initiates events such as adenosine-5'-triphosphate (ATP) exhaustion, ionic imbalances, acidosis, oxidative stress, and the generation of proteases, all of which can result in damage to brain tissue, cerebral blood vessel endothelium, and the blood brain barrier (BBB; Sandoval & Witt, 2008). Ischemia also initiates inflammatory responses and is associated with increased systemic and local release of tumor necrosis factor alpha (TNF α), interleukin-1 (IL-1), and IL-6. These increases in cytokine levels precede BBB damage and contribute to endothelial activation characterized in part by increased expression of intracellular adhesion molecule (ICAM-1), P-selectin, and E-selectin (del Zoppo et al., 2000; Huang, Upadhyay, & Tamargo, 2006). As such, ischemia creates a toxic and proinflammatory milieu for brain cells that is then exaggerated by reperfusion.

Neutrophil responses comprise one aspect of the complex inflammatory response to injury. After reperfusion, rolling neutrophils localize to the site of ischemic injury via transient neutrophil and endothelial selectin adhesion molecule interactions (P-, E-, and L-selectins and carbohydrate ligands). Following neutrophil rolling, activated neutrophils firmly adhere to activated endothelium through neutrophil CD11b and endothelial ICAM-1 interactions. Activated circulating, adhered, and migrating neutrophils augment the inflammatory responses of ischemia via release of reactive oxygen species, proteases, cytokines, and chemokines. In this way, the neutrophil-mediated inflammatory response that occurs during reperfusion extends brain cell injury after cerebral ischemia (Connolly et al., 1996; Prestigiacomo et al., 1999; Ruehl et al., 2002; Zarbock & Ley, 2009).

tMCAO Mouse Model

Because surgical manipulation can be an independent source of inflammation, a reproducible and minimally invasive mouse model of tMCAO was integral to our study of neutrophil-mediated inflammatory mechanisms of cerebral IR injury. The surgical protocol for tMCAO used in this study has been previously described (Sampei et al., 2000; W. Zhang et al., 2008). Other investigators that use a slightly different tMCAO method report a high mortality rate (25–80%; Chen, Ito, Takai, & Saito, 2008; Kitagawa et al., 1998; Tsuchiya, Hong, Kayama, Panter, & Weinstein, 2003). The most common causes of postoperative deaths are intracranial hemorrhage associated with damaged vessels incurred during filament placement, cerebral edema, excessive blood loss during surgery, and postoperative infection (Chen et al., 2008; Tsuchiya et al., 2003). In contrast, the mortality rate in the current study was remarkably low (6%). These favorable outcomes were likely due to the combined effects of the use of a transtemporal approach for Doppler placement, the use of the cautery for transecting only the ECA, the absence of tissue retractors minimizing extraneous tissue damage, and precise filament construction and placement. In this study, great care was taken to perfect filament construction; the filament size range was held to 0.21–0.25 mm.

Few if any reports of the coefficient of variation (CV, a measure of variability) are available in mouse models of tMCAO. In this study, the CV for total infarct volume was 31%, lower than the variation typically observed in tMCAO models in rats (ranging from 36% to 177%; Aspey, Taylor, Terruli, & Harrison, 2000). Strict adherence to minimally invasive, precise surgical procedures resulted in high reproducibility, low variability, and low mortality rates in this study. These findings support the argument that the observed neutrophil activation is

a function of cerebral ischemia and reperfusion and not a function of the surgical manipulation itself.

Infarct Volume, Brain Edema, and Petechial Hemorrhage

Our study uses a model of human middle cerebral artery occlusion resulting in right middle hemispheric brain damage. Figure 3 is a typical profile of middle cerebral artery occlusion. As such, the largest infarct areas were detected in the middle slices (4 and 6 mm from frontal pole) and corresponded to blood supplied by the middle cerebral artery. The total infarct volume reported in this study is consistent with those reported by others who have used a 60 min ischemia and 24 hr reperfusion tMCAO method (Chen et al., 2008; Tsuchiya et al., 2003). We also investigated whether total infarct volume correlated with the area of infarct of any one brain slice. Such correlations are important to conserve animal costs when brain tissue must be collected for various histology, protein quantification, or genetic assays. We found that total infarct volume (mm^3) strongly correlates to infarct area (mm^2) measured at 6 mm from the frontal pole.

Brain edema was evident after ischemic stroke and 24 hr of reperfusion. Edema accounted for a $7.5\% \pm 1.4\%$ increase in calculated tissue volume within the affected hemisphere when compared to the unaffected hemisphere. Edema results from increased BBB permeability and the movement of large molecules and water into brain tissue. Edema expands brain tissue enclosed within the rigid skull, thereby contributing to tissue injury. Increased BBB permeability first peaks immediately after the initiation of reperfusion due to metabolic and hyperemic effects (Sandoval & Witt, 2008). This is generally known as cytotoxic edema. A second biphasic edema, termed vasogenic edema, results from changes to BBB permeability after approximately 5 and 72 hr of reperfusion (Sandoval & Witt, 2008). Although multifactorial mechanisms are thought to contribute to both early and later phases of edema, BBB permeability changes after 6 and 48 hr of reperfusion are associated with prolonged inflammatory responses that injure the vasculature, including neutrophil-mediated responses (Zhang, Chopp, Chen, & Garcia, 1994).

To our knowledge, petechial hemorrhages have not previously been reported in mouse models of cerebral IR. It has been suggested that petechial hemorrhage after MCAO and reperfusion in rats is associated with impaired microvessel wall integrity (Nagaraja et al., 2008; R. Zhang et al., 2008). Neutrophil-mediated mechanisms contribute to impaired microvessel wall integrity after ischemic stroke and reperfusion. These mechanisms include (a) increased oxidative stress during reperfusion, (b) matrix metalloproteinase production, and (c) extravasation of leukocytes across the vessel wall (Hamann, del Zoppo, & von Kummer, 1999; Wang & Lo, 2003). Although petechial hemorrhages are associated with deleterious inflammatory events that result in increased brain injury (Wang & Lo, 2003), we did not identify a correlation between infarct volume and amount of petechial hemorrhage present within the infarct.

Systemic Leukocyte Response During Reperfusion After Ischemic Stroke

Normal mouse total WBC counts can range widely from 2 to $10 \times 10^9/\text{L}$ (Hedrich & Bullock, 2004). Total WBC counts for mouse tMCAO and sham surgical groups in this study fell within this range (Table 1). In this study, we found no differences in either WBC or differential counts in animals that underwent sham or tMCAO procedure followed by 15 min of reperfusion. After 24 hr of reperfusion, there was a significant reduction in total circulating WBCs compared to the 24-hr sham group. An observed reduction in circulating lymphocytes may account, in part, for the observed reduction of total circulating WBCs.

In mice, neutrophils normally account for 5–20% of the circulating WBC population (Hedrich & Bullock, 2004). Neutrophils rapidly mobilize from blood stores within bone marrow to enter the systemic circulation in response to injury and disease. Consistent with this known response to injury, we observed a significant increase in the percentage of circulating neutrophils after 15 min of reperfusion in the tMCAO group as compared to the sham group. Increased circulating activated neutrophils may contribute to subsequent significant neutrophil adherence to the microvasculature and migration into surrounding parenchyma, an event observed in both mouse and rat models of cerebral IR (Akita et al., 2003; Costa et al., 2006; De Simoni et al., 2004; Ishikawa et al., 2004; Ritter et al., 2000; Storini et al., 2005).

Despite the reduction in total WBC and lymphocyte counts at 24 hr of reperfusion, the percentage of systemic neutrophils was significantly increased. Significant increases in circulating neutrophils have been reported in rat models of cerebral IR at 4, 24, and 48 hr after reperfusion (Furuya et al., 2001; Ruehl et al., 2002). Our findings confirm findings from these models and signify the extended communication of injury from damaged brain tissue that results in neutrophil mobilization and response.

Exposure to isoflurane anesthesia has been reported to reduce systemic WBC and neutrophil counts in rodents (Jacobsen, Villa, Miner, & Whitnall, 2004; Marini et al., 1994), while extended (5–7 hr) use of nitric oxide with isoflurane has been reported to increase systemic WBC and neutrophil counts (Lehmberg, Waldner, Baethmann, & Uhl, 2008). The use of nitrous oxide with isoflurane in the current study likely had little effect on systemic or localized WBC and neutrophil responses as surgical times remained under 2 hr. Although statistically different, the actual difference in isoflurane delivery between sham and tMCAO groups was 0.2%. In light of the relative short surgery time and small magnitude of difference in isoflurane delivery between sham and tMCAO groups, physiologic differences between groups were likely minimal.

Neutrophil CD11b Expression

In addition to examining neutrophil mobilization during reperfusion, we investigated neutrophil surface expression of CD11b after ischemic stroke. Neutrophil surface expression of CD11b is an indicator of neutrophil activation and subsequent potential for reperfusion injury. We found a significant increase in neutrophil CD11b expression at both 15 min and 24 hr of reperfusion. Systemic neutrophil activation has the potential to contribute to reperfusion injury after ischemic stroke via the release of proinflammatory cytokines such as TNF α , proteases, and reactive oxygen species. These neutrophil products contribute to extended damage of the BBB, vascular endothelium, and eventually brain parenchyma (Sandoval & Witt, 2008).

The addition of LPS, a known neutrophil activator, further increased neutrophil activation in all groups of animals, fulfilling the role of positive control. However, this additional activation was significantly greater after 24 hr of reperfusion from ischemic stroke, suggesting that after 24 hr of reperfusion, circulating neutrophils were “primed” for additional activation. Neutrophil priming occurs when neutrophil functional responses to stimuli are amplified by earlier exposure of the cell to a priming event or agent (Swain, Rohn, & Quinn, 2002). Our findings indicate that the inflammatory environment that results from ischemic stroke and 24 hr of reperfusion acts as such a priming agent, and any additional stimulus (such as recurrent ischemia) may result in exaggerated neutrophil-mediated reperfusion injury.

In summary, the current study demonstrates that, in a well-controlled mouse model of ischemic stroke and reperfusion, WB flow cytometry is a sensitive method for assessing

systemic neutrophil activation. The results of this study confirm the findings from other animal models that systemic neutrophil activation is important in cerebral reperfusion injury. Similar measures of neutrophil activation may be useful indicators of reperfusion injury in humans with ischemic stroke but to date have not been thoroughly investigated (Caimi et al., 2001; Fiszer et al., 1998; Tsai et al., 2009). The systemic neutrophil activation described in this study has additional significance in that transgenic mouse models bred on a C57Bl/6 background are increasingly used to elucidate single mechanisms of reperfusion injury after ischemic stroke. Consequently, findings from this study are foundational for future investigations examining the damaging potential of neutrophil-mediated responses during reperfusion after ischemic stroke in mouse models. Finally, the findings of this study have relevance to the treatment strategies for stroke. Our data suggest that the damaging potential of neutrophil activation is significant and that it begins as early as 15 min and remains evident at 24 hr of reperfusion. There are currently no treatments that protect brain tissue from reperfusion injury (Ginsberg, 2009). Thus, treatment with future novel anti-inflammatory agents that attenuate neutrophil responses may have optimal effects, if delivered with early reperfusion therapies.

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Figure 1.

Experiment protocol. MCAO-R = Middle cerebral artery occlusion with reperfusion; WBC = white blood cell count.

Figure 2.

Size of cerebral ischemic injury. Infarct size (% contralateral hemisphere) assessed at 60 min of ischemia and 24 hr after reperfusion in cortex (open bar; $n = 10$), striatum (light grey bar; $n = 10$), and total hemisphere (black bar; $n = 10$). Results are mean $\pm$ SEM.

Figure 3. Infarct volume distribution. Infarct area (mm²) at 2 mm, 4 mm, 6 mm, and 8 mm from frontal pole. Inset: Correlation between infarct area (mm²) at 6 mm from the frontal pole and total infarct size (mm³). Results are mean ± SEM.

Figure 4.

Representative 2,3,5-triphenyltetrazolium chloride (TTC) image of necrosis (white area) and petechial hemorrhage (red areas located between brackets) as a result of 60 min of ischemia and 24 hr of reperfusion in a C67Bl/6 mouse model. Edema is evident from midline shift, compressed lateral ventricle (arrow), and visual increase in hemisphere size when compared to contralateral (unaffected) side. No contralateral compensatory response to brain injury was observed.

Figure 5.

Systemic neutrophils are activated at 15 min (A) and at 24 hr of reperfusion (B) after ischemic stroke. Total fluorescence intensity (TFI) for neutrophil surface expression of CD11b per 10,000 neutrophils. Open bars represent sham group, dark grey bars represent tMCAO group. Results are mean $\pm$ SEM. Group means were significantly different between sham and transient middle cerebral artery occlusion (tMCAO) group at 15 min ($n = 6$ in both groups) and 24 hr of reperfusion ($n = 7$ and $n = 10$, respectively; $*p < .05$).

Figure 6.

Systemic neutrophil priming occurs at 24 hr of reperfusion after ischemic stroke but not after 15 min of reperfusion. A. Neutrophil activation after lipopolysaccharide (LPS) incubation in whole blood of sham and transient middle cerebral artery occlusion (tMCAO) groups after 15 min of reperfusion ($n = 6$ in both groups). B. Neutrophil activation after LPS incubation in whole blood of sham and tMCAO groups after 24 hr of reperfusion ($n = 7$ and $n = 10$, respectively). Total fluorescence intensity (TFI) for neutrophil surface expression of CD11b per 10,000 neutrophils was quantified. Open bars represent sham group, dark grey bars represent tMCAO group. Results are mean $\pm$ SEM. Group means were significantly different between sham and tMCAO group at 24 hr of reperfusion ($^{\#}p < .001$).

Table 1

Physiological and Surgical Variables in C57Bl/6 Animals Subjected to Sham or Transient Middle Cerebral Artery Occlusion (tMCAO) Procedure

Variable	Sham (<i>n</i> = 13)	tMCAO (<i>n</i> = 16)	<i>p</i> Value
Temperature (°C)	36.2 ± 0.3	36.0 ± 0.3	.15
Total surgical time (min)	96 ± 1.4	117 ± 2.0	<.001 [#]
Isoflurane (%)	1.6 ± 0.05	1.4 ± 0.04	<.001 [#]
Reduction of rCBF during ischemia (%) ^a	NA	83.2 ± 7.4	NA
Return of rCBF during reperfusion (%) ^a	NA	91.9 ± 15.6	NA

Note. rCBF = relative cerebral blood flow. Data are expressed as mean ± SEM.

^aCompared to baseline rCBF measures.

¹
p < .05.

Table 2

Total White Blood Cell and Differential Blood Cell Counts (Mean $\pm$ SD) in C57Bl/6 Animals Subjected to Sham or Transient Middle Cerebral Artery Occlusion (tMCAO) Surgery Followed by 15 min or 24 hr of Reperfusion

Blood Cell Counts	15 min of Reperfusion				24 hr of Reperfusion			
	N (Sham/tMCAO)	Sham	tMCAO	p Value	N (Sham/tMCAO)	Sham	tMCAO	p Value
Total WBC count ($\times 10^9/L$)	7/5	4.0 $\pm$.4	5.4 $\pm$ 2.5	.61	7/10	4.7 $\pm$ 0.3	2.6 $\pm$.3	.001 [#]
Lymphocytes (%)	7/5	76.64 $\pm$ 2.5	66.0 $\pm$ 4.1	.04 [*]	7/9	81.1 $\pm$ 1.3	72.4 $\pm$ 3.7	.048 [*]
Neutrophils (%)	7/5	22.8 $\pm$ 1.9	32.7 $\pm$ 3.7	.03 [*]	7/9	16.4 $\pm$ 1.3	25.5 $\pm$ 2.8	.019 [*]
Monocytes (%)	7/5	1.9 $\pm$.58	2.2 $\pm$ 0.3	.75	7/9	0.9 $\pm$ 0.3	2.4 $\pm$ 0.8	.12

Note. WBC = white blood cell. Data are expressed as mean $\pm$ SEM.

^{*} $p < .01$.

[#] $p < .05$.