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Neutrophil elastase and neurovascular injury following focal stroke and reperfusion

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ABSTRACT

Neutrophil elastase (NE) degrades basal lamina and extracellular matrix molecules, and recruits leukocytes during inflammation; however, a basic understanding of the role of NE in stroke pathology is lacking. We measured an increased number of extravascular NE-positive cells, as well as increased levels of tissue elastase protein and activity, following transient middle cerebral artery occlusion (tMCAo). Both pharmacologic inhibition of NE with ZN200355 (ZN), and genetic deletion of NE, significantly reduced infarct volume, blood–brain barrier disruption, vasogenic edema, and leukocyte–endothelial adherence 24 h after tMCAo. ZN also reduced infarct volume in MMP9-null mice following tMCAo. There were, however, no reductions in infarct volume or vasogenic edema in NE-null mice in two models of permanent middle cerebral artery occlusion. Our findings confirm the involvement of NE in neurovascular stroke pathology, when reperfusion allows neutrophils access to vulnerable brain, with pharmacologic or genetic inhibition of NE being both neuro- and vasculo-protective in this setting.

Leukocytes play a critical role in post-ischemic inflammation and secondary neurovascular injury in most tissues, including brain (for review, see: Man et al., 2007; Wang et al., 2007). Mechanistically, this results from oxidative injury secondary to the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-associated respiratory burst and/or proteolytic injury from the release of destructive enzymes. Both of these events can occur independent of leukocyte transmigration across endothelium into parenchyma (Shapiro 2002). With respect to the protease pathway, the 33-kDa serine proteinase elastase (E.C. 3.4.21.37) is stored in a biologically active form within primary neutrophil granules. Neutrophil elastase (NE) is important in host defense (Belaaouaj et al., 1998), and degranulation stimuli during inflammation can include the paracrine action of platelet activating factor and interleukin-8 released from endothelial cells (Henriksen and Sallenave, 2008). NE degrades structural matrix proteins (e.g. elastin, collagens, laminins, and fibronectin), resulting in increased vascular permeability and hemorrhage in non-cerebral tissues (Ishikawa et al., 2003; Houtz et al., 2004).

In conjunction with membrane degradation, NE also regulates leukocyte adhesion and diapedesis/transmigration in peripheral vascular beds in response to pro-inflammatory stimuli (Woodman et al., 1993; Young et al., 2004). NE-mediated degradation of endothelial junction proteins, in addition to the aforementioned proteolysis of matrix proteins, "clears a path" for the transmigrating leukocytes during diapedesis (Hermant et al., 2003; Ionescu et al., 2003). In brain, exogenous delivery of NE, following either intracarotid infusion (Nagy et al., 1998) or intracerebral injection (Armao et al., 1997), increases blood–brain barrier (BBB) permeability, suggesting that BBB tight junctions, basal lamina, and perhaps even cortical endothelial cells are vulnerable to NE-mediated proteolysis during pathological conditions associated with cortical inflammation.

Studies of non-CNS tissues using pharmacologic inhibitors of NE highlight the specific role played by NE in ischemia–reperfusion pathology. NE inhibition reduces leukocyte adherence and extravasation in intestinal and myocardial ischemia (Zimmerman and Granger, 1990; Tiefenbacher et al., 1997). Post-ischemic vasogenic edema in the rabbit lung (Kishima et al., 1998), as well as in rat hindlimb (Welbourn et al., 1991) and liver (Okajima et al., 2004), can be attenuated by NE inhibitors. Few studies, however, have investigated endogenous NE in stroke, despite evidence of significant neutrophil accumulation following cerebral ischemia (Wang et al., 2007; Gelderblom et al., 2009). One study by Shimakura et al. (2000) showed that the continuous infusion of an NE inhibitor, ONO-5046, reduced post-ischemic vasogenic edema and infarct volume 24 h following permanent middle cerebral artery occlusion in rats. ONO-5046 has also been shown to protect hippocampal neurons following forebrain ischemia (Matayoshi et al., 2009). While these findings are consistent with NE mediating post-ischemic neurovascular dysfunction, more

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specific studies on leukocyte dynamics and BBB disruption, particularly in transient focal ischemia models, using both pharmacologic and genetic approaches, are needed to begin to systematically assess the deleterious effects of NE in the ischemic brain.

The present investigation was undertaken, in mice, to identify the potential post-stroke pathophysiologic roles of neutrophil-derived elastase. We hypothesized that vascular and parenchymal exposure to NE would contribute to infarct severity and vasogenic edema, secondary to promoting leukocyte adherence and attendant BBB disruption, and that NE-mediated pathology would only occur in areas of transient ischemia, where reperfusion allows neutrophils access to vulnerable regions. By cross-validating genetic and pharmacologic approaches, we obtained causal data confirming the involvement of NE in neurovascular pathology following cerebral ischemia/reperfusion.

Materials and methods

Transient and permanent focal cerebral ischemia

The Animal Studies Committee at Washington University School of Medicine approved all experimental methods and animal care procedures in accordance with NIH guidelines. Occlusion of the middle cerebral artery was performed in adult male Swiss–Webster/ND4 (SW/ND4), 129SvEv (wild-type; WT), and NE−/− mice or MMP9−/− mice on a 129SvEv background, at 10–16 weeks of age, as described previously (Gidday et al., 2005). Pilot studies were conducted in SW/ND4 and WT mice to establish transient middle cerebral artery occlusion (tMCAo) durations that led to roughly similar, moderate-sized infarcts and that maximized 24-h animal viability for subsequent analysis of leukocyte adherence and BBB integrity. Monitoring of relative cerebral blood flow (CBF) changes during and after ischemia in the final experimental groups revealed no potential strain-dependent effects (Majid et al., 2000). Matched protocols were then conducted in a blinded, randomized fashion in drug-treated or mutant mice of the same strain. During all surgical procedures, core body temperatures in mice were monitored by a rectal probe and maintained at 37 °C using a thermo-regulated heating pad.

For tMCAo, animals were anesthetized with chloral hydrate (350 mg/kg) and xylazine (4 mg/kg; 0.001 ml/g i.p.), and a blunted 6.0-gauge nylon suture was threaded 9.0–10.5 mm through the left common carotid to the origin of the middle cerebral artery (MCA; Miller et al., 2001). Interruption of blood flow to the MCA was confirmed using transcranial laser Doppler flowmetry (TSI, Inc.). The MCA was considered successfully occluded with a >80% drop in blood flow versus baseline, and successfully reperfused with a >50% blood flow return versus baseline at 10 min of reperfusion. Following suture withdrawal, mice were kept in a warmed incubator until recovered from anesthesia and fully ambulatory. A sham tMCAo group, in which the animals were anesthetized, and the intraluminal suture was placed anterogradely in the carotid but not advanced to the ostium of the MCA, was also studied. All mice subjected to tMCAo underwent serial neurobehavioral examinations performed in blinded fashion at the end of ischemia and at 24 h after reperfusion (Table 2). Each animal was assigned a neurological deficit score of 0–4, with 0 being no observable deficits and 4 being an inability to walk spontaneously (Gidday et al., 2005).

Permanent middle cerebral artery occlusion (pMCAo) was performed in two different ways in 129 SvEv WT and matched NE−/− mice. In one group, surgical procedures were identical to the tMCAo model, but after the suture was placed at the origin of the MCA, it was secured tightly in place and remained blocking the ostium of the MCA for the 24-h duration of the experiment. In another group of WT and NE−/− mice, a more distal occlusion of the MCA was performed via a craniotomy, with electrocoagulation of the surface presentation of the MCA as described previously (Majid et al., 2000; Miller et al., 2001). As with tMCAO, consequent reductions of >80% of distal baseline cortical blood flow were confirmed using Doppler flowmetry immediately following occlusion, and animals were excluded from the pMCAo group if they did not meet this criterion. Post-operative recovery procedures in these mice were the same as indicated above.

For experiments assessing the effect of pharmacologic inhibition of NE in SW/ND4 and NE−/− mice, the selective, peptidic, trifluoromethyl ketone-based NE inhibitor ZN200355 (ZN; 10 mg/kg; Mehta et al., 1994; AstraZeneca) was dissolved in physiologic saline for intraperitoneal (ip) injection. We chose to administer 10 mg/kg of ZN at both 0 h and 4 h of reperfusion, and additionally at 22 h of reperfusion in some of the leukocyte–endothelial adherence studies, based on previously-published pharmacokinetics (Williams et al., 1991; Mehta et al., 1994; Tiefenbacher et al., 1997).

Elastin-Congo red degradation assay

SW/ND4 mice underwent a 90-min tMCAo and were sacrificed 12, 24, or 48 h following reperfusion. Animals were sacrificed via cervical dislocation and the brain was immediately removed and flash-frozen. Tissue samples of the ipsilateral hemisphere were homogenized in extraction buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM CaCl2, and 0.05% Brij-35; Zhang and Gottschall, 1997). Total protein concentration was determined using a DC protein assay kit (BioRad), as per manufacturer's instructions. Equal amounts of protein were incubated with 1 ml (10 mg/ml) Congo Red-conjugated insoluble elastin (Elastin Products Inc) at 37 °C while rotating for 16 h. The solution was centrifuged to pellet any undigested, insoluble elastin, and the absorbance of cleaved elastin-Congo red by elastolytic activity was measured at 490 nm. Various concentrations of purified NE (Elastin Products Inc; 0–10 μg) were used in parallel to determine the concentration of elastolytic activity in the tissue samples.

Immunoblot analysis for NE

Based on protein concentration, equal amounts of protein from whole-brain cortical homogenates were separated under reducing conditions on a 15% SDS polyacrylamide gel and transferred onto Immobilon-P PVDF transfer membranes (Millipore Corp). After blocking overnight (5% nonfat dried milk, TBS, 0.1% Tween 20) at 4 °C, the membranes were incubated for 2 h with anti-NE antibody (1:6000; courtesy of Barry Starcher, University of Texas Health Sciences Center at Tyler), followed by 1 h of incubation with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:25,000; Jackson ImmunoResearch). After washing, immunoreactive proteins were detected by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham Pharmacia) and subsequent autoradiography.

Immunohistochemistry and confocal microscopy

Either under naïve conditions or 24 h following tMCAo, animals were transcardiac perfused (20 ml PBS followed by 40 ml 4% paraformaldehyde), and brains were removed, cryoprotected, and sectioned on a cryostat at a 10-μm thickness. At time of histology (McCandless et al., 2008), sections were brought to room temperature, rinsed in dH_2O, hydrated in 10 mM phosphate-buffered saline (PBS), and blocked (10% goat serum, 0.1% Triton, 10 mM PBS). Sections were washed and exposed to primary antibody (rat anti-mouse CD31, 1:50, BD Biosciences; rabbit anti-mouse NE, 1:100, courtesy of Dr. Barry Starcher, University of Texas Health Center at Tyler) overnight. The following day, sections were washed (3 × 15 min, 0.2% FSG, 10 mM PBS), blocked, and probed with secondary antibody (Alexa 488 goat anti-rat 1:300 and Alexa 568 goat anti-rabbit, 1:300, Molecular Probes) for 1 h. Sections were rinsed, stained for 5 min with ToPro3, and coverslipped with Immu-mount (Thermo Scientific). All images