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Interleukin-6 mediates enhanced thrombus development in cerebral arterioles following a brief period of focal brain ischemia☆

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ABSTRACT

Objective: The cerebral microvasculature is rendered more vulnerable to thrombus formation following a brief (5.0 min) period of focal ischemia. This study examined the contribution of interleukin-6 (IL-6), a neuroprotective and prothrombotic cytokine produced by the brain, to transient ischemia-induced thrombosis in cerebral arterioles. *Approach & results:* The middle cerebral artery of C57BL/6 J mice was occluded for 5 min, followed by 24 h of reperfusion (MCAo/R). Intravital fluorescence microscopy was used to monitor thrombus development in cerebral arterioles induced by light/dye photoactivation. Thrombosis was quantified as the time of onset of platelet aggregation on the vessel wall and the time for complete blood flow cessation. MCAo/R in wild type (WT) mice yielded an acceleration of thrombus formation that was accompanied by increased IL-6 levels in plasma and in post-ischemic brain tissue. The exaggerated thrombosis response to MCAo/R was blunted in WT mice receiving an IL-6 receptor-blocking antibody and in IL-6 deficient (IL-$6^{-/-}$) mice. Bone marrow chimeras, produced by transplanting IL-$6^{-/-}$ marrow into WT recipients, did not exhibit protection against MCAo/R-induced thrombosis. *Conclusions:* The increased vulnerability of the cerebral vasculature to thrombus development after MCAo/R is mediated by IL-6, which is likely derived from brain cells rather than circulating blood cells. These findings suggest that anti-IL-6 therapy may reduce the likelihood of cerebral thrombus development after a transient ischemic attack.

1. Introduction

The effects of ischemia, with or without reperfusion, on the brain have been extensively characterized. These studies have revealed the influence of a variety of factors on the brain injury response to an ischemic episode, with the magnitude and duration of insult representing major determinants of the injury response. Ischemic insults of long duration typically elicit severe tissue injury characterized by neuronal cell death, blood–brain barrier dysfunction and edema, which collectively result in brain infarction and a potentially debilitating stroke (Jung et al., 2010). Ischemic insults of shorter durations are better tolerated by the brain and often result in little or no lasting brain damage and neurological impairment. There is also a large body of evidence demonstrating that prior exposure of the brain to a transient ischemic insult of short duration confers a protective phenotype that allows brain tissue to tolerate a subsequent and more severe ischemic insult (Kirino, 2002; Gidday, 2006). The phenomenon of ischemic preconditioning (or ischemic tolerance) has been attributed to an increased expression of protective genes and the production of neuroprotective agents that render the brain more resistant to ischemic injury. While neurons are generally considered the primary cellular target of the ischemic preconditioning (IPC) response in brain (Gidday, 2006), there is evidence that the protection is also evidenced in different cellular components of the cerebral microvasculature, including endothelial cells, and is manifested as improved blood brain-barrier function, decreased endothelial adhesiveness to circulating leukocytes, and enhanced angiogenesis (Kirino, 2002; Gidday, 2006). A recently described deleterious consequence of a brief, transient ischemic insult on the cerebral vasculature is accelerated thrombus development, which is more pronounced in arterioles than in venules (Tang et al., 2014). The enhanced thrombogenesis occurs despite evidence for prolonged coagulation and bleeding times after IPC (Chen et al., 2005; Kim et al., 2008), and the thrombosis response is not altered by treatment with anti-platelet agents such as aspirin, clopidogrel, and dypiridamole (Tang et al., 2014). The nature and origin of the prothrombogenic stimulus that mediates this deleterious response to transient cerebral ischemia remain unclear.

Interleukin-6, one of the major cytokines produced by the central nervous system (Erta et al., 2012; Gadient and Otten, 1994; Schöbitz et al., 1993), exerts a variety of biological actions that impact on the injury and repair responses of the brain to an ischemic insult (Tuttolomondo et al., 2008). Potential beneficial effects of the cytokine include stimulation of neurogenesis and angiogenesis, inhibition of apoptosis, and

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blood–brain barrier stabilization (Jung et al., 2011; Erta et al., 2012). In contrast to these neuroprotective and neurotrophic effects, IL-6 has been reported to exert pro-inflammatory, pro-oxidative, and prothrombogenic actions (Spychalowicz et al., 2012; Senchenkova et al., 2013). A net protective effect of IL-6 in ischemic stroke is suggested by some animal studies that show an exaggerated brain injury response to mild transient ischemia in both IL-$6^{-/-}$ mice (Herrmann et al., 2003; Gertz et al., 2012) and in obese (ob/ob) mice treated with an IL-6 neutralizing antibody (Terao et al., 2008). Ischemic stroke and transient ischemic attacks in humans are associated with significant increases in plasma IL-6 concentration, and the results of several clinical studies have revealed the cytokine to be a strong predictor of brain injury and clinical outcome (Hoshi et al., 2005; Orion et al., 2008). Similar increases in plasma, as well as brain tissue, IL-6 concentration (or mRNA) have been described in animal models of ischemic stroke (Chapman et al., 2009; Offner et al., 2009; Terao et al., 2008; Gertz et al., 2012).

Studies in other organ systems and different pathological conditions have revealed that IL-6 is also a potent prothrombogenic cytokine (van der Poll et al., 1994; Mutlu et al., 2007; Yan et al., 2014). Anti-IL-6 therapies have been reported to reduce thrombus development in animal models of deep vein thrombosis (Wojcik et al., 2011) and in the microvessel thrombosis associated with experimental colitis (Senchenkova et al., 2013). The cytokine is known to promote a variety of responses that favor coagulation/thrombosis, including thrombocytosis secondary to stimulation of thrombopoiesis (Zhang et al., 2013), induction of platelet-leukocyte aggregation (Yan et al., 2014), and platelet activation (Peng et al., 1994; Yan et al., 2014). The cytokine also exerts an influence on the coagulation system, as evidenced by its ability to enhance the expression of tissue factor, fibrinogen, factor VIII, and von Willebrand factor, and to increase thrombin generation. IL-6 also reduces inhibitors of hemostasis such as anti-thrombin and protein S (Tuttolomondo et al., 2008). These prothrombotic actions of IL-6, coupled to the well-described elevation in plasma and tissue IL-6 concentration that occurs in response to brain ischemia (Hoshi et al., 2005; Orion et al., 2008), suggest that it is a viable candidate mediator of the prothrombogenic phenotype that is assumed by the cerebral microvasculature in response to transient cerebral ischemia. Hence, the overall goal of this study was to assess, using both immunologic and genetic approaches, the contribution of IL-6 to the accelerated thrombus formation elicited in cerebral arterioles following a brief ischemic episode. IL-6 deficient bone marrow chimeras were also used to identify the likely source of IL-6 in mediating the ischemia-induced thrombogenesis.

2. Materials and methods

2.1. Animals

Male (23–33 g body weight) C57BL/6 J and IL-$6^{-/-}$ (B6.129S6-$IL6^{tml\ Kopf}$, C57BL/6 J background) mice were purchased from Jackson Laboratory (Bar Harbor, ME). A total of 123 mice were used in the study. The distribution of mice in the different experimental groups is outlined in the figure legends. The experimental procedures were approved by the Institutional Animal Care and Use Committee at LSU Health Sciences Center and are in compliance with National Institutes of Health guidelines. All efforts were made to minimize animal distress and the number of animals used.

2.2. Induction of transient cerebral ischemia (MCAo/R)

Mice were anesthetized with 150 mg/kg ketamine and 7.5 mg/kg xylazine. Core body temperature was monitored and maintained at 36 ± 0.5 °C using a heating blanket during surgery and until recovery from anesthesia. Transient focal cerebral ischemia was induced using the intraluminal filament method, as described previously (Tang et al., 2014). Briefly, a blunted 6–0 silicone-coated monofilament (Doccol Corporation, CA) was introduced into the common carotid artery through an arteriotomy below the carotid bifurcation and advanced along the internal carotid artery into the Circle of Willis until mild resistance was felt, indicating that the filament had entered the anterior cerebral artery and blocked the origin of middle cerebral artery (MCA). Following MCA occlusion for a period of 5 min, the filament was withdrawn, allowing for brain reperfusion for a period of 24 h. We have previously demonstrated that the 5 min ischemia/24 reperfusion (MCAo/R) protocol does not yield significant sustained neurological deficits nor does it produce detectable brain infarction (Tang et al., 2014). Sham mice were subjected to the same procedure without arteriotomy and monofilament insertion.

2.3. Intravital videomicroscopy

Following 24 h of reperfusion (after MCA occlusion), the mice were prepared for intravital microscopic observation of the brain, as previously described (Tang et al., 2014). Briefly, the mice were anesthetized, a craniectomy was performed and the animal was placed on the stage of an upright fluorescent microscope (BX51WI; Olympus, Japan) and allowed to equilibrate for 30 min. Visualization of individual cerebral microvessels and the induction of thrombus formation were achieved using a 40× water immersion objective lens (LUMPlan FI/IR 40×/0.80×; Olympus). A silicon-intensified target video camera (C-2400-08, Hamamatsu, Japan) or a charge-coupled device video camera (XC-77; Hamamatsu, Japan) projected the images onto a monitor (Trinitron PVM-2030; Sony, Japan), which was connected to a video timer (Time-Date Generator WJ-810; Panasonic, Japan) to record time and date. The images were recorded on a DVD recorder (SR-MV50; JVC, Wayne, NJ). The diameters of the selected microvessels were measured by video analysis software (Image J software version 1.37; NIH, US) on a personal computer (G4 Macintosh; Apple, CA).

2.4. Light/dye induced thrombosis

A 10 ml/kg body weight dose of 5% fluorescein isothiocyanate dextran (FITC; 150,000 molecular weight; Sigma-Aldrich, US) was injected into the femoral vein cannula and allowed to circulate for 10 min before photoactivation. Photoactivation of FITC (excitation, 495 nm; emission, 519 nm) was initiated by exposing 100 μm vessel (arteriole) length to epi-illumination with a 175-W xenon lamp (Lamda LS, Sutter, US) coupled with a fluorescein filter cube (HQ-FITC; Chroma Technology, US). The excitation power density was calibrated daily (ILT 1700 Radiometer, SED033 detector; International Light Technologies, US) and maintained within 1% of 0.17 W/cm^2, as previously described (Tang et al., 2014). During continuous epi-illumination, thrombus formation was visualized and quantified in both venules and arterioles (diameters: 20–40 μm) by determining the time of onset of platelet deposition/aggregation (onset time) and the time to complete blood flow cessation (cessation time). Epi-illumination of a vessel under study was discontinued once blood flow ceased. The results of both onset time and cessation time were averaged from 2 to 4 induced thrombi in each arteriole of the same mouse.

2.5. IL-6 in plasma and brain tissue

A cytometric bead array (Mouse soluble protein master buffer kit; BD Biosciences USA) was used to measure the concentration of IL-6. Briefly, plasma and supernatants of left and right hemisphere brain tissues were collected at 24 h after MCAo/R. For these experiments, mice were transcardially perfused (to flush the cerebral vasculature) with 1 × phosphate buffered saline (PBS). The samples were processed and analyzed with the cytometric beads as per the manufacturer's instructions. The concentration of IL-6 was expressed as pg/ml in plasma or pg/g brain weight for the tissue samples.