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Differential effects of aging and sex on stroke induced inflammation across the lifespan

Bharti Manwani, Fudong Liu, Victoria Scranton, Matthew D. Hammond, Lauren H. Sansing, Louise D. McCullough *

Department of Neurology, University of Connecticut Health Center, Farmington, CT, USA
Department of Neuroscience, University of Connecticut Health Center, Farmington, CT, USA

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ABSTRACT

Aging and biological sex are critical determinants of stroke outcome. Post-ischemic inflammatory response strongly contributes to the extent of ischemic brain injury, but how this response changes with age and sex is unknown. We subjected young (5–6 months), middle aged (14–15 months) and aged (20–22 months), C57BL/6 male and female mice to transient middle cerebral artery occlusion (MCAO) and found that a significant age by sex interaction influenced histological stroke outcomes. Acute functional outcomes were worse with aging. Neutrophils, inflammatory macrophages, macrophages, dendritic cells (DCs) and microglia significantly increased in the brain post MCAO. Cycling females had higher $Gr1^{-}$ non-inflammatory macrophages and lower T cells in the brain after stroke and these correlated with serum estradiol levels. Estrogen loss in acyclic aged female mice exacerbated stroke induced splenic contraction. Advanced age increased T cells, DCs and microglia at the site of injury, which may be responsible for the exacerbated behavioral deficits in the aged. We conclude that aging and sex have differential effects on the post stroke inflammatory milieu. Putative immunomodulatory therapies need to account for this heterogeneity.

Introduction

Stroke is the fourth leading cause of death and the leading cause of adult disability in the US. Sex differences in stroke risk have been well documented. These have been predominantly attributed to the protective effects of estrogen in pre-menopausal women (Lisabeth and Bushnell, 2012; Reeves et al., 2008). Yamori et al. replicated this sexual dimorphism in the laboratory using young stroke prone spontaneously hypertensive rats which showed a significant survival advantage in ovary-intact females (Yamori et al., 1976). Importantly, the Framingham Heart Study reported that although women have lower risk of stroke than men, this epidemiology reverses with age leading to a disproportionately higher risk in elderly women compared to age matched men (Petrea et al., 2009). Disparities not only exist in stroke risk with aging but are also seen in stroke outcomes. Elderly females have more severe strokes, poorer recovery and more disability after stroke (Appelros et al., 2009; Fukuda et al., 2009; Lai et al., 2005; Niewada et al., 2005), in part due to the older age at which women experience their first stroke. These sex disparities in aged populations have been confirmed in preclinical models of ischemic stroke (F. Liu et al., 2009). Aging is the most important non-modifiable risk factor for stroke, and the majority of stroke patients are over 65 years of age (Go et al., 2013; Rothwell et al., 2004). Despite this, most pre-clinical stroke studies exclusively examine the response to stroke in young male animals (Feigin et al., 2003). This is in part due to the difficulty of performing surgeries in aging animals, the high animal costs and poor survival rates in aged animals (Liu and McCullough, 2011). Most researchers also exclude female animals due to the variability that can occur with cyclical hormone changes with estrus, which is well known to affect histological damage after injury (Carswell et al., 2000; Zucker and Beery, 2010). Overall, there has been a paucity of stroke studies in females and aging animals, leading to limitations in the translational relevance of potential therapeutics identified in experimental models.

Cerebral ischemia has profound effects on both the central and peripheral immune response. Stroke activates resident microglia and also leads to the recruitment of blood derived leukocytes into the brain (Huang et al., 2006; Iadecola and Anrather, 2012). This recruitment process is modulated by several cell adhesion molecules and cytokines which when induced, act upon the vascular endothelium to increase the expression of ICAM-1, P-selectin, and E-selectin, leading to further local accumulation and adhesion of leukocytes (Danton and Dietrich, 2003; Elkind, 2010; Han and Yenari, 2003; Ishikawa et al., 2004). After gaining entry into the CNS (through the damaged blood brain barrier), infiltrating leukocytes release cytokines and chemokines amplifying the innate (microglial) inflammatory response in the brain and causing further injury (Huang et al., 2006; Jin et al., 2010; Wang et al., 2007). This initial inflammatory cell influx in the brain is followed

* Corresponding author at: Department of Neurology, University of Connecticut Health Center, 263 Farmington Avenue, MC1840, Farmington, CT 06030, USA. Fax: +1 860 679 1181.
E-mail address: lmccullough@uchc.edu (L.D. McCullough).

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by a systemic immunodepression (Dirnagl et al., 2007). Secondary lymphatic organs including the spleen and thymus contract after focal cerebral ischemia, potentially increasing the risk of post-stroke infections (Dirnagl et al., 2007; Elkind, 2010; Emsley and Hopkins, 2008; Offner et al., 2006a), which are linked to increased morbidity and mortality. The temporal dynamics of the specific leukocyte populations infiltrating the brain parenchyma after an ischemic event has been studied (Gelderblom et al., 2009; Stevens et al., 2002) but these studies were all performed in young male animals. Aging leads to a chronic low grade inflammatory state and an altered innate immune environment in the brain, which contributes to the development of age-related disorders including stroke (Dorshkind et al., 2009; Gruver et al., 2007; Krabbe et al., 2004; Lucin and Wyss-Coray, 2009). Enhanced astroglial response and proinflammatory cytokine production has been seen in aged animals after stroke (Dinapoli et al., 2010; Popa-Wagner et al., 2007). The interaction between immunosenescence and loss of gonadal hormone secretion with aging is complex and has not been well studied. The primary gonadal female sex hormone, estrogen, appears to be anti-inflammatory in young and pro-inflammatory in the aged brain (Johnson and Sohrabji, 2005; Liu et al., 2012b; Nordell et al., 2003; Wise et al., 2001). Thus, we hypothesized that both sex and aging would shape the post-stroke inflammatory response, leading to differences in histological and behavioral outcomes between the sexes at different time points in the lifespan. We used 5–6 month old female mice as young cycling females, 14–15 month old females as a translational model of females with irregular cycles transitioning to anovulation (perimenopausal in humans), and 20–22 month old females as a model of menopause as mice are anovulatory and persistently acyclic at this age (Nelson et al., 1982; Parkening et al., 1980). The primary objective of this study was to characterize the differential effects of sex and aging on the acute peripheral and central inflammatory response to ischemic stroke.

Materials and methods

Experimental animals

Young (5–6 months), middle aged (14–15 months) and aged (20–22 months) C57BL/6 male and female mice were kept separately in cages of two animals per cage on sawdust bedding (light cycle 12/12 h light/dark). The average weight of males was 37.7 ± 1.1 g (young males), 44.4 ± 1.56 g (middle age males) and 43.6 ± 1.89 g (aged males). Female mice weighed 32.4 ± 2.72 g (young females), 34.4 ± 0.86 g (middle age females) and 29.4 ± 1.58 g (aged females). All mice had access to chow and water ad libitum.

This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and under protocols approved by the Center for Lab Animal Care at the University of Connecticut Health Center.

Ischemia model

Animals were randomly and blindly assigned to MCAO or control groups using simple random number randomization. Only one surgeon performed the surgeries and was blinded to the age of the animals. Focal transient cerebral ischemia was induced by 60 min of middle cerebral artery occlusion (MCAO) under isoflurane anesthesia, followed by reperfusion, as described previously (McCullough et al., 2005). In mice weighing greater than 35 g, a larger diameter (0.23 mm) silicon coated suture was utilized to achieve occlusion (F. Liu et al., 2009). Rectal temperature was maintained between 36.5 and 37.5 °C during surgery through an automated temperature control feedback system. Cerebral blood flow (CBF) was measured by Laser Doppler flowmetry (LDF, Moor Instruments Ltd, England) in all animals as previously described (McCullough et al., 2005).

Neurological scores

Neurological scores were recorded at the time of reperfusion (60 min) and 24 h after MCAO surgery. The scoring used was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling as described previously (McCullough et al., 2003).

Terminal histopathology for cresyl violet staining and immunohistochemistry

Two cohorts of control and MCAO animals were sacrificed at 24 hour endpoints with pentobarbital overdose (i.p.). There was no mortality in any of the cohorts 24 h after surgery. Mice with subarachnoid hemorrhages and/or tumors in the brain were excluded from the study (n = 2 (1 in MA and 1 in aged) and 2 (both in aged) respectively). One cohort was used for cresyl violet staining and immunofluorescence studies and the second for flow cytometry studies. In the cresyl violet cohort, n = 6–8/group, blood was collected from the right ventricle of the heart using heparinized syringes at sacrifice. Transcardial perfusion was performed with cold PBS followed by 4% paraformaldehyde. Brain, spleen and uteri were collected. Splenic and uterine weights were recorded and quantified as mg/g of body weight (pre-operative weight for mice subjected to MCAO). All the weights were taken by a single observer blinded to age and MCAO. The brains were fixed for 24 h and placed in cyroprotectant (30% sucrose), frozen and then sliced into 30-μm free-floating sections on a freezing microtome; every eighth slice was stained by cresyl violet stain to evaluate infarct volumes. The images were digitalized and infarct volumes were measured using computer software (Sigma scan Pro5) as previously described (F. Liu et al., 2009). The infarct volumes were calculated as percentage of contralateral hemispheric structure and corrected for edema using Swanson's method (Swanson et al., 1990).

Immunohistochemistry (IHC)

Immunohistochemical studies were performed on 30-μm brain sections as described previously (Liu et al., 2010; Manwani and McCullough, 2011; Manwani et al., 2011). Briefly, brain slices were mounted onto gelatin-coated slides, allowed to air dry and then blocked in 0.1 M phosphate buffer (PB) with 0.3% TritonX-100 (sigma) and 10% goat serum (PBTGS) for an hour. Primary antibody (Iba1, Wako, USA) was added overnight, washed with PBTGS followed by incubation with secondary antibody (1:1000) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1:1000, Invitrogen, Carlsbad, CA). Secondary antibody (1:1000, goat anti-rabbit conjugated to Alexa-488) was removed with three consecutive washes in PBTGS, 0.1 M PB, and 0.05 M PB respectively. Images were acquired with immunofluorescence confocal microscopy using Zeiss image acquisition software (Zeiss Axiovert 200 M). Brain slices were taken at the same distance from bregma (0.5 mm anterior to bregma) and three 20× fields/animal (n = 3–4 animals/group) were analyzed in the penumbral area of the infarct. Iba1 positive cells were counted using MacBiophotonics ImageJ software with DAPI (nuclear stain). The average of the total number of cells/field of view was used for statistical analysis as described previously (Liu et al., 2010; Manwani et al., 2011).

Estradiol ELISA

Blood samples collected at the time of sacrifice were centrifuged at 6000 rpm for 10 min at 4 °C to yield serum for hormone detection. Serum was stored at −80 °C until use. Enzyme-linked Immunoassay (ELISA) for 17β-estradiol (BQ, San Diego, CA) was utilized following the manufacturer's protocol.