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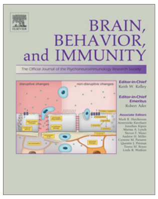

Full-length Article

Genetic deletion of galectin-3 enhances neuroinflammation, affects microglial activation and contributes to sub-chronic injury in experimental neonatal focal stroke

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The pathophysiology of neonatal stroke and adult stroke are distinct in many aspects, including the inflammatory response. We previously showed endogenously protective functions of microglial cells in acute neonatal stroke. We asked if galectin-3 (Gal3), a pleotropic molecule that mediates interactions between microglia/macrophages and the extracellular matrix (ECM), plays a role in early injury after transient middle cerebral occlusion (tMCAO) in postnatal day 9–10 mice. Compared to wild type (WT) pups, in Gal3 knockout pups injury was worse and cytokine/chemokine production altered, including further increase of MIP1α and MIP1β levels and reduced IL6 levels 72 h after tMCAO. Lack of Gal3 did not affect morphological transformation or proliferation of microglia but markedly attenuated accumulation of CD11b$^+$/CD45$^{med\text{-}high}$ cells after injury, as determined by multi-color flow cytometry. tMCAO increased expression of αV and β_3 integrin subunits in CD11b$^+$/CD45^{low} microglial cells and cells of non-monocyte lineage (CD11b$^-$/CD45$^-$), but not in CD11b$^+$/CD45$^{med\text{-}high}$ cells within injured regions of WT mice or Gal3−/− mice. αV upregulated in areas occupied and not occupied by CD68$^+$ cells, most prominently in the ECM, lining blood vessels, with expanded αV coverage in Gal3−/− mice. Cumulatively, these data show that lack of Gal3 worsens subchronic injury after neonatal focal stroke, likely by altering the neuroinflammatory milieu, including an imbalance between pro- and anti-inflammatory molecules, effects on microglial activation, and deregulation of the composition of the ECM.

1. Introduction

Perinatal arterial ischemic stroke is common—occurring in at least 1 in 2300 live term births—and produces significant morbidity and severe long-term neurological and cognitive deficits, including cerebral palsy and neurodevelopmental disabilities (Nelson, 2007; Nelson and Lynch, 2004; Raju et al., 2007). More than half of all children with cerebral palsy are born at term. There is now ample evidence that the mechanisms of ischemic injury differ greatly between the immature brain and adult brain (review in Fernandez-Lopez et al., 2014; Yager and Ashwal, 2009), including neuroimmune responses (Hagberg et al., 2015). Neuroinflammation plays a major modulatory role in the pathogenesis of stroke in the adult, both detrimental and beneficial (Iadecola and Anrather, 2011; Markowska et al., 2010; Shichita et al., 2014; Szalay et al., 2016). In perinatal stroke and hypoxic-ischemic encephalopathy (HIE), neuroinflammation also plays a key modulatory role (Hagberg et al., 2015) but the underlying signaling mechanisms are distinct, as demonstrated in mice with genetic manipulations of individual inflammatory mediators (Doverhag et al., 2008; Hedtjarn et al., 2005; Woo et al., 2012). The differing responses to stroke between neonates and adults are likely due to the CNS immaturity, including cells within the neurovascular unit, astrocyte and pericyte coverage of the brain vasculature, as well as microglial immaturity and the still present physiological neuronal programmed cell death during early postnatal brain development.

Historically, in adult stroke, microglia were viewed as purely injurious, in part due to production of inflammatory mediators and reactive oxygen species (reviewed in Vexler and Yenari, 2009). However, we discovered that after neonatal arterial stroke microglia protect neurovascular integrity, engulf and remove neuronal debris, and curb injury (Faustino et al., 2011; Fernandez-Lopez et al., 2016). Microglial cells can exert protection via many signaling mechanisms, including communication of microglia with the extracellular matrix (ECM) and direct interaction with

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endothelial cells. Our comparative analysis of the endothelial transcriptome following acute neonatal and adult stroke demonstrated marked differences in the expression of various ECM proteins produced in uninjured brain regions and distinct changes in injured regions (Fernandez-Lopez et al. 2012).

Galectin-3 (Gal3) has been demonstrated to modulate cell-cell interactions within the ECM (Danella Polli et al., 2013) and to play a modulatory role in models of adult stroke (Lalancette-Hebert et al., 2007; Lalancette-Hebert et al., 2012; Yan et al., 2009) and neonatal hypoxia-ischemia (H-I) (Doverhag et al., 2010). Considering that Gal3 is a pleotropic molecule that can exert context-dependent changes in the brain (Krzeslak and Lipinska, 2004) and exhibit brain maturation-dependent effects (Pasquini et al., 2011), in this study we focused on the role of genetic Gal3 deletion on sub-chronic injury after neonatal stroke. In a recently developed neonatal mouse arterial focal stroke model, a transient middle cerebral artery occlusion (tMCAO) model in postnatal day 9 pups (P9–P10), we show that genetic deletion of Gal3 affects microglial activation, alters the ECM-integrin communications and extends injury during the sub-chronic phase.

2. Materials and methods

All research conducted on animals was approved by the University of California San Francisco Institutional Animal Care and Use Committee and followed in accordance to the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services). Animals were given ad libitum access to food and water; housed with nesting material and shelters, and kept in rooms with temperature control and light/dark cycles.

2.1. Transient middle cerebral artery occlusion (tMCAO)

A transient 3 h MCAO was achieved by inserting a 6-0 coated filament into the internal carotid artery of male and female P7 rats, as previously described (Derugin et al., 2005; Derugin et al., 1998). Reperfusion of the MCA was achieved by the retraction of the filament. The pups were then returned to the dam until sacrifice.

Male and female P9–P10 C57Bl/6 (WT) and galectin-3 knockout (Gal3−/−) mice (on C57Bl/6 background; colony founders purchased from Jackson Laboratories, Bar Harbor, Maine) were anesthetized and subjected to 3 h tMCAO or sham surgery, followed by 24 or 72 h reperfusion as originally described for P7 rats (Derugin et al., 2005; Derugin et al., 1998) and modified for P9–P10 mice (Woo et al., 2012).

2.2. BrdU labeling, histological evaluation and immunofluorescence

To assess cell proliferation, mice were given Bromo-2-deoxyuridine (BrdU; Millipore) at 50 mg/kg of body weight by intraperitoneal injection (i.p.) twice daily with an interval of 10–12 h at 24 h, and 48 h, and one injection at 4 h before sacrifice at 72 h after MCAO.

Pups were deeply anesthetized with Euthasol (100 mg/kg; Virbac) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4). Brains were post-fixed in 4% PFA overnight at 4 °C, cryoprotected in 30% sucrose in 0.1 M PBS at 4 °C for 48 h, frozen and cut on cryostat (12 μm thick, 348 μm apart). The sizes of contralateral and ipsilateral hemispheres and the size of injured regions in 6 consecutive Nissl-stained coronal sections were traced by two investigators, one of them blinded to brain identity. Tissue loss was calculated as volumetric ratio of the remaining ipsilateral hemisphere compared to contralateral hemisphere. Injury volume is shown as ratio of injured region compared to the ipsilateral hemisphere.

For immunofluorescence, sections were blocked in 20% normal goat serum (NGS) in PBS containing 0.25% TritonX-100 followed by overnight incubation at 4 °C with rat anti-mouse Gal3 (gift from Dr. Jasna Kriz), rabbit polyclonal anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:200; Wako), rat anti-mouse Cluster of Differentiation 68 (CD68, 1:100; Bio-Rad), rat anti-BrdU (1:200; Abcam), chicken anti-BrdU (1:500; Abcam), rabbit polyclonal anti-alphaV (αV, 1:300, Abcam), rabbit polyclonal anti-glucose transporter 1 (Glut-1, 1:500; Millipore), monoclonal anti-glial fibrillary acidic protein (GFAP; 1:300; Millipore), or monoclonal anti-Neuronal Nuclei (NeuN; 1:100; Millipore) following 1 h incubation with fluorochrome conjugated secondary antibodies raised in goat (1:500; Life Technologies). Some slides were co-stained with Alexa 647-conjugated *Griffonia simplicifolia* isolectin B_4 (IB4; 1:150; Life Technologies) and with DAPI. Slides were coverslipped with Prolong Gold and mounted. Images were captured in four fields of view (FOV) in the peri-focal and ischemic core regions in the caudate and in the cortex, and in the corresponding contralateral regions using a Zeiss Axio Imager. Z2 microscope (Zeiss) equipped with Volocity Software (PerkinElmer). The number of $Iba1^+$, $CD68^+$ and $BrdU^+$ cells per FOV, mean and total surface area and volume, were measured using custom-made threshold and size-exclusion protocols created in Volocity software, as we described (Faustino et al., 2011). The density, length, surface, volume of $Glut\text{-}1^+$ vessels was determined using automated protocols for signal intensity threshold (Faustino et al., 2011).

2.3. Brain cell isolation and Multi-color Flow cytometry

Samples for multi-color flow cytometry were prepared as we described (Li et al., 2015), with modifications. Deeply anesthetized mice were transcardially perfused with 10 ml of Ca^{2+}/Mg^{2+}-free Hank's Balance Salt Solution (HBSS) to eliminate peripheral cells, meninges were removed, and the cortices of injured and matching contralateral regions were dissected on ice and enzymatically digested to obtain single brain cells using Papain-containing Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany). Myelin was removed with myelin-conjugated magnetic beads and was separated from the cell suspension by passing it through LS columns placed on a magnetic rack (Miltenyi Biotec) (Li et al., 2015).

Isolated cells were plated at a density of 2×10^5 cells per well (96-well V-bottom plate, Falcon), blocked for 15 min with CD16/32 (1:70; Biolegend) in FACS buffer containing 2% fetal bovine serum to prevent unspecific binding by antibodies. Cells were washed once in FACS buffer and stained with a mixture of antibodies (30 min, 4 °C, protected from light), as we described (Li et al., 2015). The following antibodies conjugated to fluorochromes were used: anti-CD45-Pacific Blue (1:2000; Biolegend), anti-CD11b-APC-Cy7 (1:2000; Biolegend), anti-Ly-6C-APC (1:800; eBioscience), anti-CD51(αV)-PE (1:800; BD Pharmingen), and anti-CD61(β3)-AF647 (1:800; BD Pharmingen). Following antibody incubation cells were washed once with FACS buffer. The gating strategy was based on live single cells stained with Live/Dead Yellow reactive dye (Life Technologies). BD compensation beads (BD Bioscience) were used for compensation. All samples were run on BD LSRII flow cytometer (BD Bioscience) and data analysis was performed using FlowJo software (Tree Star).

2.4. Western blot

Tissue from injured and matching contralateral regions was collected from deeply anesthetized mice transcardially perfused with HBSS and flash frozen in 2-methyl-butane under dry-ice. Tissue was mechanically disrupted in lysis buffer (Cell Signaling)