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AGE EXAGGERATES PROINFLAMMATORY CYTOKINE SIGNALING AND TRUNCATES SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION 3 SIGNALING FOLLOWING ISCHEMIC STROKE IN THE RAT

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Abstract—Neuroinflammation is associated with glial activation following a variety of brain injuries, including stroke. While activation of perilesional astrocytes and microglia following ischemic brain injury is well documented, the influence of age on these cellular responses after stroke is unclear. This study investigated the influence of advanced age on neuronal degeneration, neuroinflammation, and glial activation in female Sprague–Dawley rats after reversible embolic occlusion of the middle cerebral artery (MCAO). Results indicate that in comparison to young adult rats (3 months), aged rats (18 months) showed enhanced neuronal degeneration, altered microglial response, and a markedly increased expression of proinflammatory cytokines/chemokines following MCAO. In addition, the time-course for activation of signal transducers and activators of transcription 3 (STAT3), the signaling mechanism that regulates astrocyte reactivity, was truncated in the aged rats after MCAO. Moreover, the expression of suppressor of cytokine signaling 3 (SOCS3), which is associated with termination of astrogliosis, was enhanced as a function of age after MCAO. These findings are suggestive of an enhanced proinflammatory response and a truncated astroglial response as a function of advanced age following MCAO. These data provide further evidence of the prominent role played by age in the molecular and cellular responses to ischemic stroke and suggest that astrocytes may represent targets for future therapies aimed at improving stroke outcome.

Key words: astrocyte, microglia, middle cerebral artery occlusion, suppressor of cytokine signaling 3, interleukin 6, neuroinflammation.

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Abbreviations: ANOVA, analysis of variance; CCL2, chemokine (C-C motif) ligand 2; CNTF, ciliary neurotrophic factor; CT-1, cardiotropin-1; DPBS, Dulbecco's modified phosphate buffered saline; GFAP, glial fibrillary acidic protein; IL-6, interleukin-6; JAK, janus kinases; MCAO, middle cerebral artery occlusion; mRNA, messenger ribonucleic acid; OSM, oncostatin M; PCR, polymerase chain reaction; SE, standard error; SOCS3, suppressor of cytokine signaling 3; STAT, signal transducers and activators of transcription; TNFα, tumor necrosis factor alpha; tPA, tissue plasminogen activator.

Treatment of acute ischemic stroke injury is hampered by the inability to translate successful animal studies into clinically effective therapies. Despite considerable research interest in specific cardiovascular risk factors for stroke, such as hypertension, hypercholesterolemia, and diabetes, data from the Framingham Heart study demonstrate that age is the single greatest risk factor for stroke (Grossi, 2008). Aging results in enhanced basal expression of proinflammatory cytokines and these same proinflammatory mediators often are associated with neural injury-related activation of microglia and astroglia. Taken together, the aging, inflammation and glial activation phenotypes serve as the basis for the "inflam-aging" hypothesis (Salvioli et al., 2006; Franceschi et al., 2007; Giunta, 2008). According to this hypothesis, increased inflammation during the aging process results from dysregulation of the immune system and a progressive inability to properly handle pathological stimuli (Chung et al., 2001; Giunta, 2008). Studies on aging confirm that proinflammatory cytokines in the interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) families are participants in the complex relationship between aging and chronic morbidity (Giunta, 2008). Proinflammatory cytokines in the IL-6 family, also known as neuropoietic cytokines (Bauer et al., 2007), preferentially activate janus kinases (JAK) and the signal transducers and activators of transcription (STAT) pathways (Hirano et al., 2000). Through JAK2/STAT3 signaling, these cytokines activate target genes involved in immune responses, differentiation, survival, apoptosis, and proliferation. Activation of the JAK2/STAT3 pathway is associated with trauma and toxicant-induced astroglial activation (Sriram et al., 2004; Sofroniew, 2009) as well as the acute injury response following stroke (Planas et al., 1996; Yamashita et al., 2005; Satriotomo et al., 2006; Xie et al., 2007; Shyu et al., 2008). Taken together, these observations raise the possibility that aging may affect the proinflammatory and astrocytic response to stroke. Therefore, in the present study, we examined the effects of age on proinflammatory cytokine expression and STAT3 activation during the acute phase of injury following middle cerebral artery occlusion (MCAO) and tissue plasminogen activator (tPA) reperfusion. We found that the aged rat displayed an enhanced proinflammatory and an attenuated astrocytic response to stroke, findings with implications for future therapies.

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EXPERIMENTAL PROCEDURES

Chemicals and animals

All chemicals used in this study were of molecular biology grade and purchased from Sigma Chemical (St. Louis, MO, USA), unless otherwise noted. Human recombinant tPA was kindly gifted by Genentech (South San Francisco, CA, USA). Female Sprague–Dawley rats [(3–4 months) and (18–20 months)] were received from Hilltop Animal Laboratory (Scottdale, PA, USA) and housed under 12 hr light/12 hr dark conditions with food and water available *ad libitum*. All procedures involving rats abided by the West Virginia University Animal Care and Use Committee.

MCAO procedure

Rats from both age groups were randomly divided into MCAO and sham surgery groups. Rats were anesthetized with inhaled isoflurane (4% induction; 2% maintenance; Halocarbon; River Edge, NJ, USA) and underwent MCAO for 2 h followed by reperfusion using human recombinant tPA (5 mg/kg i.v., femoral artery; 30% bolus and 70% infused over 30 min via syringe drive) as previously described (Dinapoli et al., 2006). Ischemia was defined as a perfusion drop across the MCA region of >80% as determined by laser Doppler and successful reperfusion was denoted as a return to >80% of baseline perfusion rate by 30 min after tPA administration.

Tissue preparation and immunoblot analyses

At 6, 24, and 72 h following MCAO, rats (n=3 rats/group) were euthanized by focused microwave irradiation (3 kW for 1.5 s) to preserve steady-state phosphorylation (O'Callaghan and Sriram, 2004; Scharf et al., 2008), using a microwave applicator (Muromachi Kikai, Inc; Tokyo, Japan). Brains were removed from skulls, cortical hemispheres separated on a cold plate, weighed, homogenized in 10 volumes of hot (85–95 °C) 1% SDS, and stored at −80 °C until used. Total protein was determined by bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as standard. Activation of the STAT3 pathway was assessed by quantifying $pSTAT3^{tyr705}$ using immunoblot analysis with detection of fluorescent signals using an infrared fluorescence scanner (Licor Biosciences; Lincoln, NE, USA). Primary antibodies used in this study were rabbit anti-STAT3 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-phospho-$STAT3^{tyr705}$ (pSTAT3; 1:500) (Cell Signaling Technology, Inc., Beverly, MA, USA). Following incubation with primary antibodies, blots were washed with phosphate buffered saline with 0.1% Tween 20 (1×15 min; 2×5 min) and incubated with fluorescent-labeled anti-rabbit and anti-mouse IgG antibodies (1:2500) for 1 h. Antibody specificity was confirmed by immunoblots of the tissue homogenates.

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (PCR) amplification

Total RNA from the ipsilateral and contralateral hemispheres were isolated from young adult and aged rats (n=4 rats/group) following MCAO using Trizol® reagent (Invitrogen; Carlsbad, CA, USA). Concentration and purity of RNA was determined using a biophotometer and considered for use only if A_{260}/A_{280} was between 1.8 and 2.1. Total RNA (1 μg) was reverse-transcribed to cDNA using SuperScript™ III RNase H^- and oligo $(dT)_{12\text{-}18}$ primers (Invitrogen) in a 40 μl reaction. Real-time PCR analyses of IL-6 and suppressor of cytokine signaling 3 (SOCS3) were performed at 12, 24, and 72 h following MCAO and ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), oncostatin M (OSM), leukemia inhibitory factor (LIF), TNFα and chemokine (C-C motif) ligand 2 (CCL-2) were performed at 12 h following MCAO using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Foster City, CA, USA) in combination with TaqMan® chemistry. Glyceraldeyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize for differences in amount of cDNA added to reactions. Specific primers and dual-labeled internal fluorogenic (FAM/TAMRA) probe sets (TaqMan® Gene Expression Assays) for these genes were used according to the manufacturer's recommendation (Applied Biosystems). All PCR amplifications (40 cycles) were performed in a total volume of 50 μl, containing 1 μl cDNA, 2.5 μl of the specific Assay on Demand® primer/probe mix, and 25 μl of TaqMan® Universal master mix (Applied Biosystems). Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by manufacturer (User Bulletin 2; Applied Biosystems). Changes in messenger ribonucleic acid (mRNA) expression level were calculated following normalization to GAPDH (which did not change with age) and expressed as fold change over corresponding age-matched rats (n=3).

Neuropathology

At 6, 24, or 72 h following MCAO, rats (n=3 rats/group) were anesthetized with pentobarbital sodium (65 mg/kg i.p.) and perfused transcardially with 100 ml wash solution (0.8% sodium chloride, 0.4% dextrose, 0.8% sucrose, 0.023% calcium chloride, 0.025% sodium cacodylate) followed by 150 ml perfusion solution (4.0% sucrose, 4.0% paraformaldehyde, 1.072% sodium cacodylate). Brains were removed from skull, stored in fixative overnight, and incubated serially in 10, 20, and 30% sucrose in Dulbecco's modified phosphate buffered saline (DPBS) for 24 h. Brains were cryosectioned (25 μm) in the horizontal plane and stored in DPBS with 0.1% sodium azide until used. Brain sections were incubated with Fluoro-Jade B, a fluorescent marker for localization of degenerating neurons using a slightly modified technique (Schmued and Hopkins, 2000). Free-floating sections were mounted onto microscope slides, immersed in distilled water for 1 min, 70% ethanol for 2 min, and distilled water for 2 min. Background staining was suppressed by incubation in 0.06% $KMnO_4$ for 10 min with shaking. A rinse in distilled water for 2 min was followed by immersion in staining solution (0.01% stock solution, 4 ml of stock solution diluted in 96 ml of 0.1% acetic acid) for 20 min. After staining, slides were washed 3× in distilled water for 1 min and air dried overnight. Slides were placed on a warmer at 55 °C for 5 min, cleared in xylene for 5 min and coverslipped.

Immunohistochemistry

To visualize STAT3 immunoreactivity, free floating brain sections (n=3 rats/group) were stained using a modified ABC procedure (Vector Laboratories; Burlingame, CA, USA) (Benkovic et al., 2004). Sections were treated with 10% hydrogen peroxide in DPBS for 15 min to quench endogenous peroxidases. Following 3× rinses in DPBS for 5 min, sections were incubated in a permeabilizing solution (1.8% L-lysine, 4% normal horse serum, 0.2% Triton X-100) for 30 min at room temperature. Sections were transferred directly to primary antibody solution with 4% horse serum (rabbit anti-STAT3; 1:400; Abcam; Cambridge, MA, USA) and incubated overnight at room temperature. The following day, sections were rinsed 3× in DPBS for 5 min and transferred to the secondary antibody for 2 h (anti-rabbit IgG; 1:1000; Invitrogen). Following 3× rinses in DPBS for 5 min, sections were incubated in Avidin D-HRP (1:1000; Vector Laboratories) for 1 h at room temperature; rinsed 3× in DPBS, and incubated with Nova Red (Vector Laboratories) for 5 min. Following a 5 min rinse in distilled water, sections were mounted onto microscope slides, air-dried overnight, dehydrated through a standard ethanol series, and coverslipped.

Double-immunohistochemical detection of STAT3 and glial fibrillary acidic protein (GFAP) was evaluated using sequential procedures with STAT3 staining performed first using a nickel-enhanced procedure that imparts a blue color to STAT3. The third