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THALIDOMIDE INHIBITION OF VASCULAR REMODELING AND INFLAMMATORY REACTIVITY IN THE QUINOLINIC ACID–INJECTED RAT STRIATUM

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Abstract—Effects of thalidomide administration on vascular remodeling, gliosis and neuronal viability have been studied in excitotoxin-injected rat striatum. Intrastriatal injection of quinolinic acid (QUIN) caused time-dependent changes (durations of 6 h, 1 and 7 d post-injection) in vascular remodeling. QUIN excitotoxic insult was associated with increased numbers of vessels (laminin or collagen IV markers) demonstrating considerable abnormalities in morphology, including short fragments and vascular loops. Non-lesioned striatum, with injection of phosphate buffer solution (PBS) as a vehicle, showed no evidence for vascular remodeling. A maximal extent of vascular remodeling was measured at 1 d post-QUIN and was correlated with marked increases in microgliosis (ED1 marker) and astrogliosis (glial fibrillary acidic protein [GFAP] marker) relative to control PBS injection. Double staining of laminin with ED1 and GFAP demonstrated areas of close association of glial cells with blood vessels. Treatment of QUIN-injected animals with the anti-inflammatory compound, thalidomide significantly inhibited vascular remodeling (by 43%) and reduced microgliosis (by 33%) but was ineffective in modifying extents of astrogliosis. Intrastriatal QUIN injection was associated with a marked loss of striatal neurons relative to non-lesioned control with thalidomide treatment exhibiting a significant degree of neuroprotection (24% recovery) against QUIN-induced neurotoxicity. These results suggest close links between microglial-mediated inflammatory responses and vascular remodeling, with inflammatory reactivity associated with, and contributing to, neuronal damage in excitotoxically-lesioned striatum.

Key words: vascular remodeling, excitotoxicity, quinolinic acid, thalidomide, microgliosis.

Excitotoxicity has been implicated in the pathogenesis of a number of neurological disorders with direct neuronal damage linked to activation of glutamate receptors (Mattson, 2003). However, evidence is available to suggest that inflammatory processes could also contribute to neuronal loss following excitotoxic insult (Bal-Price and Brown, 2001; Lehrmann et al., 2001; Siao and Tsirka, 2002; Ryu et al., 2004). Links between inflammatory responses and excitotoxicity could involve actions of an assemblage of proinflammatory mediators, such as oxidative radicals, which are reported to be elevated in excitotoxic brain injury (Pearson et al., 1999; Kim et al., 2000; Santamaria et al., 2003). In such cases, microglial inflammatory responses to excitotoxic insult may be a critical factor in establishing overall neuronal viability (Ryu et al., 2005).

Recent work (Ryu and McLarnon, 2008a) has demonstrated that intrastriatal injection of the excitotoxin, quinolinic acid (QUIN) induces extensive leakiness in blood–brain barrier (BBB). The increased vascular permeability of BBB was inferred from elevated levels of blood proteins and injected plasma Evans Blue dye in parenchymal regions suggesting underlying damage to blood vessels. Leakiness of BBB is also observed with other neurodegenerative conditions including Alzheimer's disease (AD) (Kalaria et al., 1998; de la Torre, 2002). Recent work has found the anti-angiogenic and anti-inflammatory compound thalidomide efficacious in restoring intactness to BBB and providing neuroprotection in $A\beta_{1\text{-}42}$-injected rat hippocampus (Ryu and McLarnon, 2008b). It was suggested that the beneficial effects of thalidomide in the AD animal model could primarily be due to inhibition of inflammatory responses; interestingly, thalidomide analogues have recently been reported as promising agents for treatment of MS patients (Contino-Pepin et al., 2009).

On the basis of previous findings of BBB leakiness in QUIN-injected brain (Ryu and McLarnon, 2008a), we posited that inflammatory processes may be inextricably linked with vascular remodeling following excitotoxic insult. Furthermore, we considered that pharmacological modulation of inflammatory responses could aid in stabilization of vascular responses and confer neuroprotection. To test these points we have examined effects of thalidomide on a spectrum of processes induced by striatal excitotoxic insult including vascular remodeling, microgliosis, astrogliosis and neuronal damage.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (Charles River, Montreal, QC, Canada) weighing 280–300 g were used in this study. The rats were housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by the University of British

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Abbreviations: AD, Alzheimer's disease; BBB, blood–brain barrier; CMC, carboxymethylcellulose; GFAP, glial fibrillary acidic protein; IL, interleukin; ir, immunoreactivity; NeuN, neuronal nuclei; NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; QUIN, quinolinic acid; RT, room temperature; TNF-α, tumor necrosis factor-α.

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Columbia Animal Care Ethics Committee, adhering to guidelines of the Canadian Council on Animal Care. All efforts were made to minimize the number of animals used and their suffering.

Administration of agents

QUIN. Animals were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Intrastriatal injection of QUIN was performed as previously described (Ryu et al., 2004). In brief, animals received unilateral injection of 1 μl QUIN (60 nmol; Sigma, St. Louis, MO, USA) over 4 min using a 10-μl Hamilton syringe fitted with a 26-gauge needle at the following coordinates: AP: +1.0 mm, ML: −3.0 mm, DV: −5.0 mm, from bregma (Paxinos and Watson, 1986). The injection syringe was left in place for an additional 4 min to allow QUIN to diffuse from the needle tip. After removing the needle, the skin was sutured and the animals were allowed to recover and then returned to their cages.

Thalidomide

Thalidomide (Biomol, Plymouth Meeting, PA, USA), an angiogenesis inhibitor (D'Amato et al., 1994; Madhusudan and Harris, 2002), was emulsified in 0.5% carboxymethylcellulose (CMC) in phosphate-buffered saline (PBS) and administered i.p. at a dose of 100 mg/kg, beginning 12 h before QUIN injection, at the time of QUIN injection, and at 12 h post-injection. Animals subjected to QUIN injection received i.p. injections of CMC vehicle. The dose and treatment protocol used in this study was well tolerated by the animals and reported to be effective in inhibition of angiogenesis (Kenyon et al., 1997; Kaicker et al., 2003). One day following QUIN injection, the animals were deeply anesthetized with sodium pentobarbital and transcardially perfused as described below.

Tissue preparation

At 6 h and 1 and 7 days after QUIN injection, the animals were deeply anesthetized with an overdose of sodium pentobarbital and then perfused transcardially with heparinized cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull and postfixed in the same fixative solution overnight and then placed in 30% sucrose for cryoprotection. The brains were then frozen in powdered dry ice and stored at −70 °C. Coronal brain sections (40 μm) were cut throughout the striatum on a cryostat and the sections were stored in cryoprotectant solution.

Immunohistochemistry

Free-floating sections from different time points were processed for the immunohistochemistry as described previously (Ryu et al., 2004). Briefly, endogenous peroxidase was quenched with 3% hydrogen peroxide in 0.1 M PBS and sections were incubated in blocking solution containing 10% normal goat serum (NGS) and 0.2% Triton X-100 in 0.1 M PBS for 30 min. The sections were then incubated at 4 °C for 24 h with the following primary antibody against neuronal nuclei (NeuN; 1:500, Chemicon, Temecula, CA, USA) for neurons and laminin (1:1000; Sigma) or collagen IV (1:1000; Chemicon) for vessels. The immunospecificity of laminin antibody for angiogenesis has been previously demonstrated (Rosenstein et al., 1998; Krum and Khaibullina, 2003). Sections were incubated at room temperature (RT) for 2 h with biotinylated anti-mouse or anti-rabbit IgG (1:200, Vector, Burlingame, CA, USA), followed by an avidin–biotin–peroxidase complex (ABC, 1:200, Vector) for 1.5 h. Reaction products were visualized with 3,3′-diaminobenzidine (DAB, Sigma) and hydrogen peroxide.

Double immunofluorescence staining

For double immunofluorescence staining, free-floating sections from different time points were blocked for 30 min with 10% NGS and incubated overnight at 4 °C with laminin (1:500; Sigma) in combination with glial fibrillary acidic protein (GFAP; 1:1000; Sigma), a marker for astrocytes or ED1 (1:500; Serotec, Oxford, UK), a marker for activated microglia/macrophage. Sections were then incubated in a mixture of Alexa Fluor–conjugated 488 anti-rabbit IgG (1:100; Molecular Probes, Eugene, OR, USA) and Alexa Fluor–conjugated 594 anti-mouse IgG (1:100; Molecular Probes) at RT for 2 h in the dark. Sections were then washed, dried, coverslipped, and examined under a Zeiss Axioplan 2 fluorescent microscope (Zeiss, Jena, Germany) using a DVC camera (Diagnostic Instruments, Sterlings Heights, MI, USA) with Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

Histological analysis

The extent of neovascularization induced by intrastriatal QUIN injection was evaluated by measuring the area of laminin immunoreactivity (ir) of vessels in the striatum. The four striatal sections were obtained from QUIN-injected brain at the level of AP: +1.4, +1.2, +1.0, and +0.8, according to the atlas of Paxinos and Watson (1986). Immunostained striatal section images were digitized and analyzed using the image analysis program NIH version 1.57 (Wayne Rasband, NIH) as described previously (Ryu et al., 2004; Ryu and McLarnon, 2008a). The areas of vascularization (termed vascular remodeling) were estimated as the mean area of four sections containing laminin or collagen IV ir and expressed as laminin or collagen IV–ir area. The density of NeuN, GFAP, and ED1 immunoreactive cells in the striatum was evaluated by immunoreactive cell body counting using an ×400 objective magnification under a Zeiss Axioplan 2 fluorescent microscope (Zeiss) with Northern Eclipse software (Empix Imaging) and expressed as the number of cells/mm^2. All quantitative analyses were carried out in a blinded manner and four matched sections from each animal were used.

Statistical analysis

All data are expressed as means±SEM. Statistical significance of differences for group comparisons was assessed using Student *t*-test or one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test when applicable (GraphPad Prism 3.0; San Diego, CA, USA). Significance was set at $P<0.05$.

RESULTS

Vascular remodeling in QUIN-injected striatum

This component of study used two markers for changes in vasculature: laminin, an integral component of extracellular matrix and an associated protein, collagen IV, a constituent of basement membrane in endothelial cells. The time-dependent changes in laminin ir, induced by QUIN intrastriatal injection (6 h, 1 and 7 days), are presented in Fig. 1A. The changes in laminin ir with excitotoxic insult contrast with the low levels of the marker in nonlesioned PBS control (time point of 1 day, upper left panel; Fig. 1A). QUIN striatal injection was associated with a marked increase in laminin ir (up to 1 day) followed by a subsequent decrease in laminin staining at the longest time point post-QUIN injection (7 days). The QUIN-induced laminin ir showed evidence for abnormalities in blood vessels, including the presence of vascular loops and short fragments of vessels. We refer to the overall patterns of vascular changes as remodeling of vessels, which include increased numbers of laminin (+)ve vessels exhibiting abnormal morphological properties. Quantification for the