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TRAUMATIC INJURY ELICITS JNK-MEDIATED HUMAN ASTROCYTE RETRACTION *IN VITRO*

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Abstract—Brain injury causes dysfunction of the blood–brain barrier (BBB). The BBB is comprised of perivascular astrocytes whose end-feet ensheath brain microvascular endothelial cells. We investigated trauma-induced morphological changes of human astrocytes (HA) and human cerebral microvascular endothelial cells (hCMEC/D3) *in vitro*, including the potential role of mitogen-activated protein kinase (MAPK) signal-transduction pathways. HA or hCMEC/D3 were grown on flexible culture membranes and subjected to single traumatic injury normalized to 20%, 30% or 55% membrane deformation. Cells were assayed for morphological changes (i.e. retraction) and MAPK phosphorylation and/or expression (c-Jun NH_2-terminal kinase (JNK)1/2, extracellular signal-regulated kinase (ERK)1/2, and p38). HA retraction was rapidly elicited with a single traumatic injury (55% membrane deformation; $p < 0.01$). Morphological recovery of HA was observed within 2 h ($p < 0.05$). Traumatic injuries increased phospho-JNK1/2 ($p < 0.05$) in HA, indicating MAPK activation. Pre-treatment of HA with structurally distinct JNK inhibitors (25 μM), either SP600125 or SU3327, reduced JNK phosphorylation ($p < 0.05$) and trauma-induced HA retraction ($P < 0.05$). In contrast to HA, traumatic injury failed to induce either morphological changes or MAPK activation in hCMEC/D3. In summary, traumatic injury induces JNK-mediated HA retraction *in vitro*, while sparing morphological changes in cerebral microvascular endothelial cells. Astrocyte retraction from microvascular endothelial cells *in vivo* may occur after brain trauma, resulting in cellular uncoupling and BBB dysfunction. JNK may represent a potential therapeutic target for traumatic brain injuries.

Key words: trauma, human, astrocytes, brain microvascular endothelial cells, blood–brain barrier.

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disabilities (Lam and Mackersie, 1999; Ducrocq et al., 2006; Faul et al., 2010; Shlosberg et al., 2010; Morrison et al., 2013). The psychological, emotional and financial toll of TBI is enormous. Despite the immense consequences of TBI, the pathophysiology is still unclear and the treatment is largely supportive.

The blood–brain barrier (BBB) regulates the local brain microenvironment (DeVries et al., 1997; Abbott et al., 2006, 2010), and is composed primarily of microvascular endothelial cells and astrocytes (Bradbury, 1985; DeVries et al., 1997). The endothelial cells line the luminal side of microvasculature and regulate the exchange of substances between the blood and the brain (Abbott et al., 2006). The basolateral side of the cerebral endothelium is surrounded by a basement membrane, ensheathed by astrocyte end-feet (Bradbury, 1985; Abbott et al., 2006; Mathiisen et al., 2010). Cellular coupling between astrocytes and microvascular endothelial cells is essential for normal brain functioning and relies on close cellular proximity (Mathiisen et al., 2010). Astrocytes signal the endothelium for increased cerebral blood flow (CBF) to supply oxygen and nutrients, and removal of toxic by-products (Lok et al., 2007; Wang and Bordey, 2008). This coupling between metabolic demand and supply requires release of vasodilating/vasoconstricting factors from astrocytes to the endothelium and thereby regulates local CBF (Takano et al., 2006). Uncoupling of the astrocyte–endothelium unit by TBI (Castejon, 1998; Pascucci, 1988; Lam and Mackersie, 1999), may lead to the dysregulation of CBF (Lok et al., 2007; Wang and Bordey, 2008).

Cellular responses following injury are typically mediated by the signal transduction mitogen-activated protein kinase (MAPK) pathways, including extracellular

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Abbreviations: BBB, blood–brain barrier; CBF, cerebral blood flow; CFDA, 5,6-carboxyfluoroscein diacetate; EnGS, Endothelial Cell Growth Supplement; ERK, extracellular signal-regulated kinase; HA, human astrocytes; hCMEC/D3, human immortalized cerebral microvascular endothelial cells; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; TBI, traumatic brain injury.

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signal-regulated kinase (ERK), the c-Jun NH_2-terminal kinase (JNK) and the p38 MAPK (Kyriakis and Avruch, 2012). MAPK activation regulates cytoskeletal stability (Kramerov et al., 2012), leading to changes in cellular morphology (Mori et al., 2002a,b; Dhandapani et al., 2003; Jayakumar et al., 2008; Kyriakis and Avruch, 2012).

Our aims were to investigate morphological changes in human astrocytes (HA) and human cerebral microvascular endothelial cells (hCMEC/D3) to traumatic injuries *in vitro*, including MAPK activation. To this end, we employed the Cell Injury Controller II, which induces a biaxial stretch injury to cultured cells that mimics a mechanical strain experienced by tissues during rapid rotational acceleration/deceleration head movement (Ellis et al., 1995). The advantage of this injury model is the ability to apply highly controlled, repeatable injuries to cultured cells that mimic relevant forces and tissue deformation created by trauma *in vivo* (Ellis et al., 1995; Rzigalinski et al., 1997; Slemmer et al., 2002).

EXPERIMENTAL PROCEDURES

This study was approved by the Western University Ethics Review Board and Biohazard Committee.

Cell cultures

Primary HA were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). HA were grown on fibronectin-coated plates and Bioflex® culture well (Flexcell International Corp, Hillsborough, NC, USA) in VascuLife® cell culture medium supplemented with recombinant human epidermal growth factor (5 ng/mL), L-glutamine (5 mM), ascorbic acid (50 μg/mL), fetal bovine serum (4%), penicillin (100 IU/mL) and streptomycin (100 μg/mL). HA were grown in 37 °C with 5% CO_2 until cell monolayer reaches confluency. Passages 2–7 were used for experimentation. Human cerebral microvascular endothelial cells (hCMEC/D3) were provided by Dr. Pierre-Oliver Couraud (INSERM, Paris, France) (Weksler et al., 2005) hCMEC/D3 were grown on fibronectin-coated Bioflex® culture well plates (Flexcell International Corp, Hillsborough, NC) in VascuLife® Endothelial Cell Growth Supplement (EnGS) cell culture medium enriched with recombinant human EnGS (0.2%), recombinant human epidermal growth factor (5 ng/mL), L-Glutamine (10 mM), hydrocortisone hemisuccinate (1.0 μg/mL), heparin sulfate (0.75 μg/mL), ascorbic acid (50 μg/mL), fetal bovine serum (5%), penicillin (100 IU/mL) and streptomycin (100 μg/mL). hCMEC/D3 were grown in 37 °C with 5% CO_2 until the cell monolayer reaches confluency.

Traumatic injury

Traumatic injuries *in vitro* were achieved with the Cell Injury Controller II (Virginia Commonwealth University Medical Center, Richmond, VA, USA), an electronically controlled pneumatic device capable of inducing regulated and repeatable trauma to cell culture. The Cell Injury Controller II delivers a pulse of controlled gas pressure to deform a flexible membrane fitted at the bottom of a commercially available 24-well Bioflex® culture plate *in vitro* (Flexcell International Corp, Hillsborough, NC, USA) (Ellis et al., 1995). In our experimental approach, the Cell Injury Controller II delivered a 50-ms burst of gas into the culture well to induce a direct traumatic injury with rapid membrane deformation. The Cell Injury Controller allows for delivered pressure adjustments to produce varying degrees of traumatic injury: 20% deformation with a 1.8-peak PSI; 35% deformation with a 2.7-peak PSI; and 55% deformation with a 4.0-peak PSI. During the deformation of the membrane, cultured cells adhering to the membrane surface experienced a biaxial stretch to simulate a rotational, acceleration/deceleration-type trauma *in vitro* (Ellis et al., 1995).

Morphological analyses

Cell cultures were initially screened for morphological changes using bright phase microscopy. Bright-phase microscopic images of HA were captured prior to traumatic injury, then at 30, 60, and 120 min post-injury. Cellular retraction (i.e. surface area of visible culture membrane exposure) in the microscopic images were quantified using AxioVision Microscope software (Carl Zeiss, Oberkochen, Germany). As only HA demonstrated appreciable changes after traumatic injuries, HA were pre-loaded with 20 μM 5,6-carboxyfluoroscein diacetate (CFDA; Molecular Probes, Eugene, OR, USA) in Vasculife® astrocyte culture medium for 30 min at 37 °C. Following 2× wash with warm phosphate-buffered saline (PBS), HA received either no injury or injury, and were further incubated for another 30 min. Subsequently, images of the uninjured and injured CFDA-stained HA monolayer were captured by fluorescent microscopy (Zeiss Axiovert 200 M Inverted Microscope; Carl Zeiss, Oberkochen, Germany). Four random fields of view for each treatment were captured at 10× magnification. Cell monolayer retraction was assessed by quantifying the percentage of cell coverage in each image measured by autothresholding the total surface area covered by cells (visible under fluorescence) using an ImageJ software.

Quantification of cell detachment and death

Culture media from triplicate wells of uninjured and injured HA were collected into microtubes and centrifuged at 20,000 g for 10 min. Pellets were each resuspended in 75 μL of cold PBS and the number of cells were counted using a Haemocytometer (Hausser Scientific, Horsham, PA, USA) and expressed as the number of cells detached per monolayer. To determine the total number of astrocytes in a confluent monolayer, confluent cells were fixed in 3% paraformaldehyde (PFA; Bioshop Canada Inc, Burlington, Canada) in PBS for 15 min and permeabilized in 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) in PBS for 10 min. Cellular nuclei were stained with Hoechst 33342 nuclear stain (Roeche, Mississauga, Canada) for 2 min and images were captured by fluorescent microscopy (Zeiss Axiovert 200M Inverted Microscope; Carl Zeiss, Oberkochen, Germany). The number of cells (with respect to the number of nuclei) in a 10,000-μm^2 field of