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A flow cytometric approach to analyzing mature and progenitor endothelial cells following traumatic brain injury

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ABSTRACT

Background: Traumatic brain injury (TBI) continues to be a major source of death and disability worldwide, and one of the earliest and most profound deficits comes from vascular damage and breakdown of the blood–brain barrier (BBB). Cerebral vascular endothelial cells (cvECs) and endothelial progenitor cells (EPCs) have been shown to play essential roles in vessel repair and BBB stability, although their individual contributions remain poorly defined.
New method: We employ TruCount beads with flow cytometry to precisely quantify cvECs, EPCs, and peripheral leukocytes in the murine cortex after controlled cortical impact (CCI) injury.
Results: We found a significant reduction in the number of cvECs at 3 days post-injury (dpi), whereas the EPCs and invading peripheral leukocytes were significantly increased compared with sham controls. Proliferation studies demonstrate that both cvECs and EPCs are undergoing cell expansion in the first week post-injury. Furthermore, analysis of protein expression using mean fluorescence intensity found increases in PECAM-1, VEGFR-2, and VE-Cadherin expression per cell at 3 dpi, which is consistent with western blot analysis.
Comparison with existing methods: Classic methods of cell analysis, such as histological cell counts, in the traumatic injured brain are labor intensive, time-consuming, and potentially biased; whereas flow cytometry provides an efficient, non-biased approach to simultaneously quantify multiple cell types. However, conventional flow cytometry that employs capped events can provide misleading results in CNS injured tissues.
Conclusions: We demonstrate that TruCount quantification using flow cytometry is a powerful tool for quantifying mature and progenitor endothelial cell changes after TBI.

1. Introduction

Every year, nearly 2 million individuals are victims of traumatic brain injury (TBI), contributing to over 30% of all injury-related deaths in the United States alone (Centers for Disease and Prevention, 2013). TBI is a multifaceted progressive disorder that can lead to profound neurological deficits that are typically initiated by external mechanical forces to the brain. Acute disruption of the vascular network leads to subdural hematoma, epidural hematoma, and/or intraparenchymal hematoma even in some mild TBI cases (Kan et al., 2012). Open head and penetrating wounds can also lead to intracranial hemorrhage as a result of vascular damage in the brain. Even though vessel damage is associated with the majority of TBI patients, our understanding of the mechanism(s) and dynamics of vessel damage, repair, and regeneration are limited.

Normal brain function is dependent on an array of vascular networks to receive an adequate supply of oxygen and nutrients. The blood vessel endothelium is composed of cerebral vascular

Abbreviations: (BABB), Benzyl alcohol, benzyl benzoate; (BBB), Blood–brain barrier; (BSA), Bovine serum albumin; (Cdh)-5, Cadherin; (CNS), Central nervous system; (cvEC), Cerebral vascular endothelial cell; (CCI), Controlled cortical impact; (cc), Corpus callosum; (Ctx), Cortex; (dpi), Days post-injury; (EC), Endothelial cell; (EPC), Endothelial progenitor cell; (EtOH), Ethanol; (FMO), Fluorescence minus one; (H&E), Hematoxylin and eosin; (Hipp), Hippocampus; (IHC), Immunohistochemistry; (i.p.), Intraperitoneal injections; (L/D), Live/Dead; (MFI), Mean Fluorescence Intensity; (PFA), Paraformaldehyde; (PECAM)-1, Platelet endothelial cell adhesion molecule; (PBS), Phosphate buffered saline; (PE), Phycoerythrin; (RIPA) buffer, Radioimmunoprecipitation assay; (SEM), Standard error of the mean; (TBI), Traumatic brain injury; (VEGFR)-2, Vascular endothelial growth factor receptor; (VE)-Cadherin, Vascular endothelial; (Th), Thalamus; (THF), Tetrahydrofuran; (Va), Volume analyzed; (YFP), Yellow fluorescent protein; (DAPI), 4',6-diamidino-2-phenylindole; (EdU), 5-ethynyl-2'-deoxyuridine.

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endothelial cells (cvECs) that form tight junctions and interact with other vascular cells, such as pericytes and astrocytes, and their basal lamina to form a selectively permeable barrier in the central nervous system (CNS) called the blood–brain barrier (BBB; Abbott et al., 2010). TBI can lead to BBB disruption associated with decreased supply of oxygen and nutrients and increased infiltration of circulating elements that includes peripheral cells (Abdul-Muneer et al., 2013). Peripheral cell infiltration can include circulating leukocytes, such as monocytes, macrophages, T-cells, B-cells, and other inflammatory cell types, but also includes circulating endothelial progenitor cells (EPCs; Das et al., 2012). Increased infiltration of bone marrow-derived EPCs are believed to have beneficial influences on vessel repair and possibly new vessel growth (Guo et al., 2009; Timmermans et al., 2009; Xue et al., 2010); however, the cellular responses of cvECs and peripheral EPCs within the changing TBI environment are complex and remain poorly defined.

Flow cytometry is a rapid, flexible, and sensitive technique that allows for detailed simultaneous measurements of diverse cell populations within specific tissues. Flow cytometry utilizes light scatter properties to measure the relative size and internal complexity of particles of interest, while fluorescently labeled dyes or antibodies are used to measure viability, DNA content, or protein expression levels (Bendall et al., 2012; Lugli et al., 2010). Less than a decade ago, flow cytometry was a method used primarily in the field of immunology (De Rosa et al., 2001; Perfetto et al., 2004), but has been adopted by multiple fields including neuroscience. In the progressive TBI pathology, flow cytometry can be used to analyze the temporal flux of multiple cell types at a given time point; however, proper identification of the target cell populations becomes challenging as a result of injury-induced environmental alterations that include cell loss, proliferation, differentiation, and infiltration. Hence, data interpretation is heavily dependent on the gating strategy (Mair et al., 2016), inclusion and exclusion markers, as well as single color and isotype controls to provide an adequate assessment of cellular dynamics in TBI tissues. Here, we demonstrate that accurate cell quantification can be achieved in the controlled cortical impact (CCI) injured mouse cortex using measurements based on capped (predetermined number) events in combination with TruCount beads and defined markers for both mature cvECs and infiltrating EPCs to demonstrate the relative contributions of each cell type to CNS injury. Combining this analysis with proliferative markers strengthens our understanding of the environmental contributions that lead to temporal differences in cell numbers.

2. Materials and methods

2.1. Animals

Generation of Cdh5-zG mice resulted from crossing Cdh5(pac)-CreERT2 (Tg (Cdh5-cre/ERT2) 1Rha, MGI: 3848982) (Sorensen et al., 2009) with Rosa zGreen reporter mice (007906 B6.Cg-Gt (ROSA) 26Sor < tm6 (CAG-ZsGreen1) Hze>/J; The Jackson Laboratory, Bar Harbor, ME). Thy-1-YFP mice were obtained from Jackson Laboratory (JAX Mice Database—003782 B6.Cg-Tg (Thy-1-YFP) HJrs/J). All procedures related to animal use and care were approved by the University of Miami Animal Use and Care Committee. Animals were housed in a 12 h light/dark cycle and food and water were supplied *ad libitum*.

2.2. Controlled cortical impact (CCI) injury

Male mice between the ages of 2–4 months were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal (i.p.) injections. Using aseptic techniques, a 5 mm craniotomy was made using a portable drill over the right parieto-temporal cortex (−2.5 mm caudal and 3 mm lateral from bregma, epicenter). The injury was generated using a 3 mm beveled stainless steel tip attached to an eCCI-6.3 device (Custom Design & Fabrication), at velocities ranging from 2 to 6 m/s, depth of 0.5 mm deep and 150 ms impact duration. The surgeries for all flow cytometry and western blot studies were performed at a velocity of 4 m/s. After CCI injury the skin was sutured using 5-0 coated vicryl sutures (Ethicon, J391) and animals were placed on a warm heating pad until they recovered from anesthesia. Surgical sham mice received only the opening and re-suturing of the skin.

2.3. Histology

Mice were anesthetized and prepared for CCI injury as described above. Animals received intracardiac perfusion with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) at 3 and 7 days post-CCI injury (dpi). Brains were post-fixed in 4% PFA overnight at 4 °C, dehydrated in 30% sucrose for 24 h and embedded in clear frozen section compound (VWR, 95057-838) for cryostat sectioning. Thirty micron cryostat sections were stained using a standard hematoxylin and eosin (H&E) histological staining. Slides were rinsed in distilled water for 5 min to remove all section embedding compound then immersed in hematoxylin for 3 min and rinsed again in running distilled water for 5 min. Slides were then immersed in eosin for 30–45 s followed by 3 min incubations of increasing ethanol (EtOH) concentrations: 70% EtOH (1X), 95% EtOH (2X), and 100% EtOH (2X). Slides were then immersed in methyl salicylate for 5 min followed by 3 times in xylene for 3 min. Slides were mounted with a xylene-based mounting medium and samples were imaged using a bright field Olympus BX50 microscope equipped with Olympus SC30 digital color camera and Olympus analysis getIT software for image capturing.

2.4. Lectin infusion

Mice were deeply anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine cocktail and intracardiac perfusion was performed with approximately ~50 mL ice cold, filtered PBS to flush blood. Mice were mildly fixed with 1% PFA and then 15 mL of DyLight 594 labeled Lycopersicon Esculentum (Tomato) Lectin (DL 1177; Vector Laboratories, Burlingame, CA), was slowly flushed through the heart followed by 50 mL of ice cold 4% PFA.

2.5. THF dehydration and tissue clearing for ultramicroscope imaging

Brains were post-fixed in 4% PFA overnight in 4 °C, protected from light and then washed in PBS for 24 h. Brains were dissected and the ipsilateral injured cortex was placed in a 30 mL glass amber packer bottle (VWR; Suwanee, GA) for the remaining steps. A four-step dehydration procedure was performed with increasing concentrations of tetrahydrofuran (THF) in distilled water: (1) 50% THF for 2 h; (2) 80% THF for 2 h; (3) 100% THF overnight (~16 h); and (4) 100% THF for 24 h. All incubation steps were done at 4 °C on a shaker. For clearing, the brains were incubated in a 1/3 benzyl alcohol (Sigma-Aldrich, 402834), 2/3 benzyl benzoate (Sigma-Aldrich, B6630) (BABB) solution for 2–4 h (on a shaker at 4 °C) prior to imaging. Cleared cortices were imaged on a LaVision BioTec Ultramicroscope based around an Olympus MVX10 zoom microscope body including light sheet excitation lines OPSL (50 mW), 488 nm, OPSL (50 mW), 561 nm and diode laser (50 mW), 647 nm. The camera used was an Andor Neo scientific low noise sCMOS camera with GFP/FITC: 525/50, TRITC/Alexa594: 620/60 and Alexa 647/Cy5: 700/80 detection channels. 3D image analysis was performed using Imaris 8.1.2 (Bitplane) software.