

Why Molecular Biomarkers of Traumatic Brain Injury May Never Work: Effects of Glymphatic Pathway Dysfunction

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Abstract

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults around the world because it has been the most misunderstood and misdiagnosed problem among the central nervous system (CNS) disorders. Around 90% of TBIs are classified as mild (mTBI). The current detection of mTBI relies heavily on an assessment of behavioral symptoms, often with delay and subject to motivation. Despite notable advances in diagnostic magnetic resonance imaging (MRI), it remains a challenging issue to precisely make early evaluation of the severity of TBI and to predict the long-term outcomes. Currently there are no molecular biomarker-based blood tests that can accurately determine the presence and the severity of TBI because at present no clinical tools are available for measuring glymphatic-derived convective bulk flow in humans. There is an urgent need to call for a concerted effort to search for sensitive and reliable biomarkers of TBI, especially mTBI. There is a growing consensus that TBI, no matter what the cause, leads to dysfunction of the blood-brain barrier (BBB), which is mainly constituted by brain microvascular endothelial cells (BMEC). Our recent preclinical studies have shown that circulating BMEC in the peripheral blood, which are independent of the glymphatic system, could be used as cell-based biomarkers for quantitative assessment of BBB injury caused by various pathogenic insults, including trauma. The vimentin-α7 nAChR pathway significantly contributes to cBMEC shedding during the pathogenesis of BBB/CNS disorders. The cell-based biomarkers cBMEC along with the single cell technology will overcome the limitations of molecular biomarkers mentioned above and make the early diagnosis of TBI.

Keywords: Traumatic brain injury; Blood brain barrier; Brain microvascular endothelial cells

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults around the world because it has been the most misunderstood and misdiagnosed problem [1,2]. About 1.7 million people are affected by TBI annually with a 3.1% (52,000 deaths) mortality rate. TBI contributes to 30.5% of all injury-related deaths in the US. WHO's predictions estimate that TBI will become the third leading cause of death in the general population by 2020 [3]. Around 75-90% of TBIs are classified as mild (mTBI), which is more common than stroke, dementia, and epilepsy [4]. mTBI is usually induced by a blow or jolt to the head and defined as a syndrome of physical, cognitive, emotional, and sleep-related symptoms [4]. The rate of mTBI is greatly underestimated because there is lack of established biomarkers or imaging modalities for diagnostic and prognostic purposes and the majority of studies are focused on the data from emergency departments [4,5]. The current detection of mTBI relies heavily on an assessment of behavioral symptoms, often with delay and subject to motivation. TBI is traditionally diagnosed using injury severity scores. The Glasgow coma scale (GCS) is the method most commonly used because of its simplicity, reproducibility, and predictive value for prognosis [6]. However, the GCS still has limitations, including medical sedation, paralysis, endotracheal intubation, and intoxication. Despite notable advances in diagnostic

computed tomography (CT) scan and MRI, it remains a challenging issue to precisely make early evaluation of the severity of TBI and to predict the long-term outcomes. These methodologies are usually unable to identify mild-to-moderate injury at the early stage, while pathophysiological changes associated with TBI may persist for several days after the injury [3,4]. During this period repetitive mTBI may result in severe consequences and trigger the development of chronic traumatic encephalopathy, which is associated with both molecular and cellular changes. Accurate early diagnosis of mTBI could facilitate development of guidelines for return to duty, work, or sports activities, and provide right interventions for preventing short- and long-term complications [4]. The emerging recognition of these challenging issues has created an urgent need to call for a concerted effort to search for sensitive and reliable biomarkers of TBI, especially mTBI.

Problematic Molecular Biomarkers of TBI

Despite the identification of hundreds of molecular markers by preclinical and clinical research over the past few decades, there is currently no blood test clinically available for objective evaluation of TBI severity. There are 14 potential fluid biomarkers for detection of TBI [7,8]. These molecular biomarkers, including S100B, UCHL1, GFAP, NSE, and Tau, have low specificity to predict brain injury [3]. Out of these, two biomarkers receiving a lot of attention are glial fibrillary acidic protein (GFAP) and S100B. Both are peripheral blood markers of astroglial injury. In published studies, the level of these two biomarkers increased in patients with TBI. S100B may be a good

predictor of brain injury severity and outcome after severe TBI [3,9]. GFAP is not found in extracerebral cells in comparison to S100B, which is also found in peripheral tissues [3,8]. S100b is released upon damage to Schwann cells, as well as peripheral tissues including chondrocytes, adipocytes and melanoma cells. Consequently, this biomarker has been found to be increased in the serum of patients with extracranial trauma in the absence of TBI [3]. A recent prospective study following a large cohort of trauma patients with and without mild to-moderate TBI demonstrated that S100b only showed 5% specificity for brain trauma and a 11% positive predictive value for brain lesions identified by CT. The same study also showed that GFAP possessed a 20% positive rate for CT-identifiable brain lesions in spite that GFAP out-performs S100 β in detecting intracranial CT lesions, particularly in the setting of extracranial fractures [10,11]. Both of these biomarkers are often obtained from cerebrospinal fluid (CSF), since CSF samples are more indicative of the biochemical changes that occur in the brain. However, CSF samples are difficult to extract, carry risks and are costly. Peripheral blood samples are safer and easier to collect than CSF. However, the concentrations of these potential TBI biomarkers in peripheral blood tend to be low. The tremendous attention and efforts could not significantly improve the diagnostic utility of molecular biomarker-based blood assays and have left many researchers offering alternative explanations for the diagnostic failure of these protein biomarkers [3].

Reasons for Failure: Effects of the Newly Discovered Glymphatic System

Why are no blood-based biomarker assays currently available for diagnosis of TBI? Several possible explanations, including insufficient assay sensitivity, proteolytic degradation, and hepatic and renal clearance, have been proposed for the diagnostic failure of these protein biomarkers [3]. The most important issue that challenges the protein biomarker-based diagnosis of TBI is not particularly clear as to how these molecular biomarkers extravasating from the BBB or neurons or glial cells end up in the systemic circulation. A mechanistic understanding of how these molecular biomarkers are produced from the brain and then released into the blood may reveal the knowledge gaps between the identification of biomarkers and the use of these molecules as diagnostic tools. Blood levels of endogenous brain proteins increase quickly following the increased blood brain barrier (BBB) permeability, no matter if it is either due to hyperosmotic disruption, endarterectomy, cerebral microvascular disease, tumor metastasis or TBI [3]. However, it remains unclear how these proteins are transported into the extracellular space and then able to move into the more distant perivascular channels before crossing the BBB. There are two different processes: diffusion and bulk flow that may contribute to the movement of solutes within the interstitial space of the brain [12]. Sykova and Nicholson demonstrated that ten hours would be taken for an albumin-sized molecule to diffuse 1 mm within the extracellular space of the brain [12]. However, molecules are further found to be cleared from the brain at the same rates no matter the size of the substance [3]. These studies suggest that molecular movements within the brain's interstitium are governed by bulk flow. In a seminal study by Illif et al, it was shown that the recently discovered the glymphatic pathway contributes to this convective bulk flow process that drives interstitial fluid from the brain parenchyma into perivenous spaces [13]. These perivascular channels then serve as a distribution center for brain-derived molecules. At least a portion of these molecules are transported across the BBB due to the receptor-mediated mechanism or pathological insult, while the rest of the

circulating molecules are moved back into the subarachnoid CSF compartment through the bulk flow. CSF is then drained into the venous circulation through arachnoid granulations, ultimately enters the lymphatic system [13]. This functional waste clearance pathway for the mammalian CNS is called the glymphatic system, which is important for the delivery of these molecules to the various points of efflux. The new glymphatic system provides the mechanistic explanation for the diagnostic failure of the molecular biomarkers [3,7]. Any factors, such as sleep deprivation, which suppress this pathway, would prevent the appearance of molecular biomarkers in the blood. More recently a number of studies have been conducted to show the effects of sleep deprivation or restriction on restoration and rejuvenation of the brain for optimal function. When sleep is deprived, the brain does not have time to perform glymphatic clearance [14,15]. A recent study showed that the appearance of injury biomarkers in the blood could be prevented by suppressing the glymphatic pathway, suggesting that the blood levels of these molecular biomarkers are not directly correlated with brain injury [4].

Can we Turn the Diagnostic Failures into Success?

Quantitative evaluation of the BBB injury has been one of the most challenging issues in the CNS disorders caused by microbial (e.g. meningitic pathogens) and non-microbial (e.g. trauma, methamphetamine and nicotine) insults [16]. There is a growing consensus that TBI, no matter what the cause, leads to dysfunction of the BBB, which is mainly constituted by BMEC. Because the brain is the most delicate organ of the body that is protected by the BBB, which constitutes the largest surface area, breakdown of this barrier follows TBI and can last from several days to years after the acute event. One of the major challenges in molecular neuroimaging approaches is the poor ability of imaging agents to cross the BBB [16]. Since the BBB mainly consists of the specific endothelial cells, called BMEC, it seems plausible that circulating BMEC (cBMEC) could be biomarkers for BBB dysfunctions. We have recently demonstrated that microbial (e.g. HIV gp41, gp120) and non-microbial factors (e.g. nicotine) could significantly cause dysfunction of the BBB in mice which correlated with increasing cBMEC as well as endothelial progenitor cells (EPC) in peripheral blood. It has been shown that similar results could be observed in patients infected with HIV-1 [17]. It is postulated that the cell-based biomarkers cBMEC/EPC along with single cell technology (SCT) will overcome the limitations of molecular biomarkers mentioned above and make the diagnosis of TBI more accurate and efficient. One of the long-sought milestones in modern biomedicine has been the development of the SCT approaches for simultaneous detection of multiple molecular markers and gene networks in single cells [18]. SCT analysis of cBMEC and EPC will overcome the limitations of single biomarkers. As such, the cBMEC-based technology represents a novel opportunity to fill an important need in assessing human brain health and to link the power of cell-based biomarkers with the challenging issues of mTBI.

The precise mechanism responsible for the pathogenic insult-mediated increase in BBB permeability and cBMEC shedding during CNS inflammation remains elusive. As shown in our previous studies, $\alpha 7$ nAChR was able to directly or indirectly upregulate proinflammatory factors (IL-1 β , IL-6, TNF α , MCP-1, MIP-1 α , RANTES, CD44 and ICAM-1), significantly enhance leukocyte transmigration into CSF and has a detrimental effect on the permeability of the BBB in the early stages of meningitic infection [19]. Calcium signaling mediated by $\alpha 7$ nAChR is the major regulatory

pathway for the CNS inflammatory response to meningoencephalitis pathogen infection and nicotine exposure. Using the $\alpha 7$ KO mouse model, we demonstrated that decreased cbMEC shedding was correlated with CNS inflammatory response (e.g. decreased leukocyte recruitment and albumin leakage into CSF) when compared to that in the wild type animals. Furthermore, we have demonstrated recently that vimentin plays an important regulatory role in the early and late events of NF- κ B signaling. The effects of vimentin blockage on gene expression could show significant inhibition of NF- κ B in the nuclei of human BMECs lacking vimentin. Our further studies demonstrated that vimentin could control NF- κ B activity by forming a complex with I κ B, NF- κ B and tubulins in the resting cells. This complex is dissociated upon the prolonged stimulation with IbeA, a meningoencephalitis virulence factor that binds to vimentin [20].

that $\alpha 7$ nAChR and other proinflammatory factors are regulated by vimentin. These findings provide insight into an element of host defense previously unknown to contribute to the BBB integrity and cbMEC shedding, but the implications of the vimentin- $\alpha 7$ nAChR pathway for the pathogenesis and therapeutics of BBB disorders and CNS inflammation remain to be explored. Both UCHL1 and S100B are shown to be involved in regulation of NF- κ B [20]. S100B, which is a ligand of Vim and calcium-binding protein, may play a bridge role in the cross-talk between Vimentin and $\alpha 7$ nAChR because it has been shown to be involved in vimentin/nicotinic receptor-mediated signaling, and NF- κ B activation [21,22]. It is likely that vimentin- and $\alpha 7$ nAChR-mediated NF- κ B signaling may be involved in regulation of both the molecular (UCHL1 and S100B) and cellular (cbMEC shedding) biomarkers during various CNS disorders (Figure 1).

Conclusion

Currently there are no molecular biomarker-based blood tests that can accurately determine the presence and the severity of TBI because at present no clinical tools are available for measuring glymphatic-derived convective bulk flow in humans. However, the cell-based biomarkers cbMEC/EPC along with the SCT approaches will overcome the limitations of molecular biomarkers mentioned above and make the diagnosis of TBI more accurate and efficient because the blood levels of cbMECs as well as EPCs positively correlate with BBB injury and host inflammatory response during CNS injury and inflammation. These findings enlighten the potential of these noninvasive cell-based biomarkers in indexing BBB injury and optimize therapeutic options.

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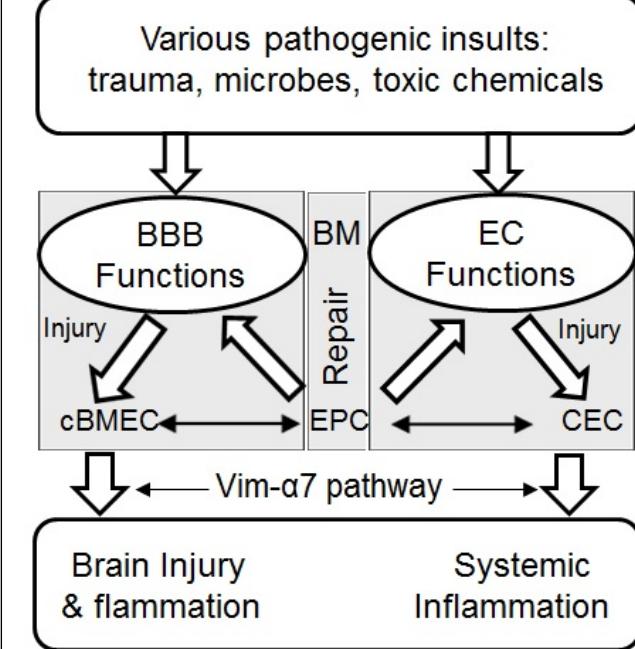


Figure 1: Role of cell-based biomarkers in physiology and pathology of the BBB. During BBB vascular turnover BMECs might be replaced by proliferation of adjacent cells or by maturation of circulating EPCs generated in the bone marrow (BM). Circulating endothelial cells (CECs) and BMECs (cbMECs) with a mature phenotype, derived from systemic and BBB vessel turnover, respectively, are increased in patients with systemic inflammation and BBB disorders. The vimentin- $\alpha 7$ (nAChR) pathway significantly contributes to cbMEC shedding during the pathogenesis of BBB/CNS disorders. The role and the frequency of BM-derived circulating EPCs may vary in different types of CNS inflammation and in different phases of BBB disorders including TBI. In addition to cbMECs and EPCs, cerebral angiogenesis might be modulated by some other specialized cells such as astrocytes and pericytes.

The vimentin head domain is essential for its interactions with I κ B/NF- κ B. These data also showed that cytoplasmic levels of vimentin, $\alpha 7$ nAChR and other signaling molecules could be significantly reduced in vimentin siRNA-transfected cells, suggesting

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