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Phage display for identification of serum biomarkers of traumatic brain injury

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HIGHLIGHTS

- Phage display is an unbiased approach to identify serum biomarkers.
- Applicable to acute CNS injuries as well as neurological disorders.
- Identification of GFAP as serum biomarker of TBI provides proof-of-concept.

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ABSTRACT

Background: The extent and severity of traumatic brain injuries (TBIs) can be difficult to determine with current diagnostic methods. To address this, there has been increased interest in developing biomarkers to assist in the diagnosis, determination of injury severity, evaluation of recovery and therapeutic efficacy, and prediction of outcomes. Several promising serum TBI biomarkers have been identified using hypothesis-driven approaches, largely examining proteins that are abundant in neurons and non-neural cells in the CNS.
New method: An unbiased approach, phage display, was used to identify serum TBI biomarkers. In this proof-of-concept study, mice received a TBI using the controlled cortical impact model of TBI (1 mm injury depth, 3.5 m/s velocity) and phage display was utilized to identify putative serum biomarkers at 6 h postinjury.
Results: An engineered phage which preferentially bound to injured serum was sequenced to identify the 12-mer 'recognizer' peptide expressed on the coat protein. Following synthesis of the recognizer peptide, pull down, and mass spectrometry analysis, the target protein was identified as glial fibrillary acidic protein (GFAP).
Comparison with existing methods and conclusions: GFAP has previously been identified as a promising TBI biomarker. The results provide proof of concept regarding the ability of phage display to identify TBI serum biomarkers. This methodology is currently being applied to serum biomarkers of mild TBI.

1. Introduction

Mild traumatic brain injury (mTBI), often referred to as a concussion for sports-related injuries, represents a major health concern (Jeter et al., 2013). mTBIs account for up to 90% of the brain injuries in the United States, affecting between 1.6 and 3.8 million people yearly, and representing a "silent epidemic" (Jordan, 2013; Langlois et al., 2006). While many cases fully resolve spontaneously, others result in long term consequences including chronic cognitive difficulties and postconcussive syndrome. Repetitive insults can result in chronic traumatic encephalopathy, a neurodegenerative dementing disorder (Butler, 2013; Carroll et al., 2004; Maroon et al., 2012; Topolovec-Vranic et al., 2011).

There is not a precise definition of mTBI, but it is generally considered to be a brief loss of consciousness (less than 30 min) or loss/alteration of neurologic function such as memory, caused by

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an interaction between biomechanical forces and the head, with negative radiology findings (Eakin and Miller, 2012; Rosenbaum and Lipton, 2012; Shultz et al., 2011). Within the mTBI category, there are a range of injury severities with 90% not resulting in a loss of consciousness (Ropper and Gorson, 2007; Rosenbaum and Lipton, 2012). Symptoms can include disorientation, confusion, amnesia, impaired concentration, sleep disturbance, irritability, anxiety, fatigue, headache, dizziness/vertigo, nausea, vacant stare, unsteady gait, impaired coordination, diplopia/blurred vision, photophobia, hyperacusis, and concussive convulsion/impact seizure (Jordan, 2013). These symptoms, however, can be associated with other conditions and thus accurate diagnosis and assessment as to when and whether mTBI has occurred is critical for proper therapeutics.

Accurate diagnosis of mTBI is particularly important in the acute stages when treatments could be most effective (Ponsford et al., 2001, 2002; Wade et al., 1998). To aid in the objective diagnosis and evaluation of mTBI, there is an urgent need for biomarkers as highlighted in NIH workshops and reviews (Jeter et al., 2013; Manley et al., 2010; Saatman et al., 2008; Zetterberg et al., 2013). The power of biomarkers is evident in cardiac injury, where cardiac troponin proteins and brain natriuretic peptide are now routinely utilized to help diagnose myocardial infarction and congestive heart failure.

Several potential cerebrospinal fluid (CSF) and serum biomarkers for TBI have been investigated, including S100β, neuron specific enolase, glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1, and spectrin breakdown products (for a recent review see Kulbe and Geddes, 2016). These biomarkers have the greatest sensitivity and specificity with severe TBI, but are less sensitive for mTBI (Agoston and Elsayed, 2012; Brophy et al., 2011; Czeiter et al., 2012; Mondello et al., 2012, 2010; Topolovec-Vranic et al., 2011). mTBI biomarkers would also be useful to assist with prediction of outcomes, evaluation of recovery and therapeutic efficacy, and provide insight into the mechanisms involved for evidence-based therapeutic interventions.

There are two major approaches to identify biomarkers—hypothesis driven and unbiased. Hypothesis-driven approaches have largely focused on proteins abundant in the cells impacted by TBI, including neurons (neuron specific enolase, ubiquitin C-terminal hydrolase-L1, spectrin breakdown products, spectrin N-terminal fragment, tau, neurofilament proteins), astrocytes (S100B, glial fibrillary acidic protein), oligodendrocytes and myelin (myelin basic protein), as well as inflammatory markers and oxidized lipids (Dash et al., 2010; Giacoppo et al., 2012; Kochanek et al., 2008; Kulbe and Geddes, 2016; Pineda et al., 2004; Sandler et al., 2010; Siman et al., 2009; Yokobori et al., 2013).

Unbiased methods to identify TBI biomarkers have included 2D proteomics. This method has been successful in identifying proteins released from degenerating cultured neurons (Guingab-Cagmat et al., 2012; Loov et al., 2013; Siman et al., 2004, 2009) and proteins differentially expressed in lysates from injured vs. uninjured rat brain (Wang et al., 2005). More recently, 2D proteomics has been applied to biofluids obtained from animal models of mTBI (Ding et al., 2015). While proteomics is powerful, it can be problematic for serum biomarker identification (Diamandis, 2004). Moreover, 2D proteomics is most applicable to medium–large proteins (Zurbig and Jahn, 2012). Other separation technologies such as liquid chromatography–mass spectrometry (LC–MS), SELDI-MS, and capillary electrophoresis–MS also have limitations including restricted mass range and low sensitivity (Zurbig and Jahn, 2012). The difficulty in detecting low molecular weight peptides and proteins is relevant to biomarker discovery, as this group includes cytokines, chemokines, peptides, and proteolytic fragments of larger proteins. This is not to suggest that 2D proteomics or related methods are not valuable. However, additional methods may identify novel and complimentary proteins/peptides as biomarkers.

To identify novel biomarkers for TBI, phage display represents a powerful, unbiased approach (Azzazy and Highsmith, 2002; Bradbury, 2010) Phage display is a method to select peptides, proteins or antibodies with specific binding properties (Bratkovic, 2010). It is most widely used to investigate protein–protein interactions, receptor- and antibody-binding sites, and for selecting antibodies against a range of antigens (Bradbury, 2010; Bratkovic, 2010; Sidhu et al., 2000). Phage display uses bacteriophages in which DNA encoding peptides or proteins are inserted into the gene encoding a coat protein of a filamentous phage such as M13 phage. M13 is a filamentous bacteriophage in which a circular single stranded DNA, 6407 nucleotides long, encodes a major coat protein (P8) and several minor coat proteins (P3, P6, P9) on the ends. A DNA encoding a peptide of interest is inserted into the P3 phage coat protein. Five copies of the protein are expressed for P3. Following infection with bacteria, new protein is synthesized and expressed on the viral particle. These foreign proteins can then bind to proteins of interest and the binding partners can be identified by sequencing.

To determine the suitability of phage display for identification of TBI serum biomarkers, we utilized the mouse controlled cortical impact (CCI) model (1 mm depth, 3.5 m/s velocity) which results in moderate neuron degeneration and cortical tissue damage (Smith et al., 1995; Saatman et al., 2006). Glial fibrillary acidic protein (GFAP) was identified as a putative serum TBI biomarker, providing proof-of-concept regarding the ability of phage display to identify serum biomarkers. The phage display methodology is currently being applied to serum obtained from rats following midline fluid percussion injury, a model of mTBI resulting in diffuse injury, to identify novel serum biomarkers of mTBI (Cao et al., 2012; McIntosh et al., 1987).

2. Materials and methods

In a proof-of-concept study, we utilized a commercial phage display library (Ph.D. 12 Phage Display Library Kit, England Biolabs, Ipswich, MA) to identify phages that preferentially bind to serum following contusive TBI. CCI was used to model contusive TBI as described previously (Madathil et al., 2013; Saatman et al., 2006). Male C57BL/6 mice, 8–10 weeks old, were anesthetized with isoflurane (3% induction, 2.5% maintenance). The head was secured in a stereotaxic frame (David Kopf Instruments, CA), and following a midline scalp incision a 5 mm diameter craniotomy was conducted over the left parietal cortex. Cortical contusion was produced using a pneumatically driven impactor (Precision Systems and Instrumentation LLC, Fairfax Station, VA) with a 3 mm diameter rounded impactor tip, 1 mm impact depth, and a velocity of 3.5 m/s. Following injury, the craniotomy was sealed with dental cement and the scalp was sutured. Body temperature was maintained at 37 °C. Control animals were not injured. Six hours following injury, animals were euthanized and blood was collected transcardially in a BD SST tube, retained 30 min for clotting, and centrifuged at 1300 × *g* for 15 min. Aliquoted serum was stored at −80 °C. One aliquot of serum (5 μl) from each of four injured animals was pooled and diluted in 1:100 in TBS. Similarly, serum aliquots from two control animals were pooled and diluted in TBS.

To identify phages selective for serum from injured mice, subtractive panning was performed using a commercial random peptide library (Ph.D.™-12 Phage Display Peptide Library Kit, New England Biolabs). The library contains greater than 2 billion independent clones that express random 12 amino acid peptides on the N-terminus of the minor coat protein P3. Pooled serum form control mice, diluted 1:100 in TBS (100 μl) was added in one well of a 96 well plate (Nunc Immuno Plate, Maxisorp surface) and 100 μl of diluted pooled injured serum was added to a second well. The