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Novel Tools and Methods

Osteopontin Is a Blood Biomarker for Microglial Activation and Brain Injury in Experimental Hypoxic-Ischemic Encephalopathy

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Abstract

Clinical management of neonatal hypoxic-ischemic encephalopathy (HIE) suffers from the lack of reliable surrogate marker tests. Proteomic analysis may identify such biomarkers in blood, but there has been no proof-of-principle evidence to support this approach. Here we performed in-gel trypsin digestion of plasma proteins from four groups of 10-d-old mice [untouched and 24 h after low-dose lipopolysaccharide (LPS) exposure, hypoxia-ischemia (HI), or LPS/HI injury; n = 3 in each group] followed by liquid chromatography-tandem mass spectrometry and bioinformatics analysis to search for HI- and LPS/HI-associated brain injury biomarkers. This analysis suggested the induction of plasma osteopontin (OPN) by HI and LPS/HI, but not by sham and injury-free LPS exposure. Immunoblot confirmed post-HI induction of OPN protein in brain and blood, whereas Opn mRNA was induced in brain but not in blood. This disparity suggests brain-derived plasma OPN after HI injury. Similarly, immunostaining showed the expression of OPN by Iba1+ microglia/macrophages in HI-injured brains. Further, intracerebroventricular injection of LPS activated microglia and up-regulated plasma OPN protein. Importantly, the induction of plasma OPN after HI was greater than that of matrix metalloproteinase 9 or glial fibrillary acid protein. Plasma OPN levels at 48 h post-HI also parallel the severity of brain damage at 7-d recovery. Together, these results suggest that OPN may be a prognostic blood biomarker in HIE through monitoring brain microglial activation.

Key words: HIE; hypoxia-ischemia; LC-MS/MS

Significance Statement

There are no reliable blood biomarkers for neonatal brain injury in hypoxic-ischemic encephalopathy (HIE) to date, and the majority of previous studies tested candidate biomarkers individually. Here we report, for the first time, proof-of-principle evidence that mass spectrometry–based quantitative proteomic methods may efficiently identify blood biomarkers in HIE. Using this method, we show that osteopontin (OPN), an integrin-binding protein secreted by activated microglia, rises in blood and correlates with severity of brain damage in experimental HIE. These data suggest that OPN may be a blood biomarker in HIE.

Introduction

Hypoxic-ischemic encephalopathy (HIE) as a result of perinatal asphyxia is a major cause of neonatal mortality and neurological disabilities, with an incidence of 1–2 per 1000 live births in the Western world and higher incidence in developing countries. Hypothermia is the current best...
therapy for HIE, but there are no simple laboratory tests to acutely monitor brain damage, which has hindered the development of better treatments (Bennet et al., 2010). Blood biomarkers are an attractive idea to overcome this obstacle, and previous studies using a candidate approach have implicated metalloproteinase 9 (MMP-9) and glial fibrillary acid protein (GFAP) as potential blood biomarkers in H1 brain injury (Bembea et al., 2011; Bednarek et al., 2012). However, quantitative proteomics has not been used to identify plasma biomarkers in HIE, despite its success in neurodegenerative disease research (Donovan et al., 2013).

To test the applicability of quantitative proteomics for the discovery of HIE biomarkers, we used in-gel trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare plasma proteins isolated at 24-h recovery from four groups of mouse neonates: unchallenged, treated with low-dose lipopolysaccharide (LPS), Vannucci hypoxia-ischemia (HI), or LPS/Hi-injured. The comparison with low-dose LPS-treated animals (which do not exhibit brain injury) is to minimize LPS-induced nonspecific immune alterations in brain damage-related biomarkers in LPS/Hi injury. Here we report the proteomics-based discovery and validation of OPN as a plasma biomarker for HIE.

OPN, also called secreted phosphoprotein 1 (SPP1), is a small integrin-binding ligand N-linked glycoprotein (SIBLING) that mediates cell–cell and cell–matrix interactions (Bellahcène et al., 2008). The basal OPN level in adult brains is low, but ischemia induces microglia/macrophages to express OPN in the peri-infarct area (Ellison et al., 1998; Gliem et al., 2015). Previous studies have shown OPN induction in rodent brains after neonatal HI, but whether there is increase of plasma OPN protein has not been evaluated (Chen et al., 2011; van Velthoven et al., 2011). Earlier studies suggested protective functions of OPN in neonatal HI, but later studies failed to confirm this finding (Albertsson et al., 2014; Bonestroo et al., 2015). Although the role of OPN in HI-injured brains remains uncertain, our results implicate plasma OPN as a prognostic biomarker for microglia activation and brain damage in HIE.

Materials and Methods

Animal surgery

The Vannucci model of neonatal HI with and without LPS preexposure (0.3 mg/kg, IP, 4 h pre-HI), and intrace- 1.5-ml PBS to remove the cerebral cortex, the resulting 2.5-mm-diameter brain slices were immediately transferred to a 37°C water bath. The slices were then rinsed in 0.125 M ice-cold PBS for 10 min, and placed in a 37°C water bath for 10 min to equilibrate the brain slices. The brain slices were then incubated in 1% Triton X-100/PBS for 30 min at room temperature to permeabilize the cells. The brain slices were then incubated in 1% normal goat serum/PBS for 30 min to block non-specific binding. The brain slices were then incubated in a 1:100 dilution of rabbit anti-OPN antibody (Abcam, Cambridge, MA) in 1% normal goat serum/PBS for 1 h at room temperature. The brain slices were then washed three times with 1% normal goat serum/PBS for 5 min each, and incubated in a 1:200 dilution of goat anti-rabbit IgG (Alexa Fluor 568, Invitrogen) in 1% normal goat serum/PBS for 1 h at room temperature. The brain slices were then washed three times with 1% normal goat serum/PBS for 5 min each, and incubated in a 1:100 dilution of DAPI (4′,6-diamidino-2-phenylindole, Invitrogen) in 1% normal goat serum/PBS for 5 min at room temperature. The brain slices were then washed three times with 1% normal goat serum/ PBS for 5 min each, and mounted on glass slides using Fluoromount-G (Southern Biotech, Birmingham, AL). The brain slices were imaged using a confocal microscope (Zeiss, Jena, Germany).
TGTTTCATC; mouse, AGCCACAGTTTACACAGCCA-CAAGG; CTGAGAAATGAGCAGTTAGTATTCCTGC.

Antibodies
The following antibodies were used for immunoblotting or immunofluorescence labeling: anti-OPN (R&D Systems, AF808), anti–cystatin C (R&D Systems, AF1238), anti–GAPDH (EMD Millipore, MAB374), anti–β-actin (Sigma-Aldrich, A5441), anti–MMP9 (Sigma-Aldrich, AV33090), anti–GFAP (EMD Millipore, AB5804), anti–Iba1 (Wako, 019-1974), anti–transferrin (Abcam, ab9538), anti–NG2 (EMD Millipore, AB5320), and anti–NeuN (EMD Millipore, MAB377). Secondary antibodies for immunocytochemistry were conjugated to Alexa Fluor 488 and 594 (Invitrogen).

Statistical analyses
All values are expressed as mean ± SD. Significance of correlations was determined by Pearson’s correlation coefficient (GraphPad Prism). p < 0.05 was considered significant. Reporting of significantly changed proteins identified by label-free quantitative proteomics relied on the false discovery rate and fold change. The statistical analysis was performed on data normalized to plasma OPN levels of unchallenged mice. These data suggest that plasma OPN may monitor a common pathological process following cerebral ischemia and HI, such as microglia activation, but is not a sensitive marker for systemic immune activation.

HI-induced plasma OPN is derived from brain microglia/macrophages
To determine the source of plasma OPN, we compared Opn mRNA levels in brain and peripheral blood mononuclear cells (PBMCs) after HI insult in mouse and rat neonates. The analysis showed 20- to 35-fold increases of Opn mRNA in brain, but minimal changes in PBMCs in animals that exhibited cerebral infarction 24 h post-HI (Fig. 2A and insets). Our data are consistent with past studies showing strong post-stroke induction of Opn mRNA in rodent brain, but not in blood (Tang et al., 2002, 2003).

To determine OPN-expressing cells, we used immunostaining to examine HI-injured brains and found strong OPN expression in Iba1+ microglia/macrophages and occasional OPN expression in NG2+ glial progenitors, but not in GFAP+ astrocytes (Fig. 2B), similar to previous reports (Ellison et al., 1998; Gliem et al., 2015). Accordingly, in vitro LPS stimulation triggered the expression of OPN by microglial cells (Fig. 2C). Moreover, ICV injection of LPS, a model of selective microglia activation, induced brain Opn mRNA and plasma OPN protein 24 h later (Fig. 2D). These results suggest that OPN is mainly produced by activated microglia and macrophages in HI-injured brain and is subsequently transported to blood as a secreted phosphoprotein.

Plasma OPN is a sensitive and predictive biomarker for brain injury in experimental HIE
We also compared protein levels of OPN, MMP-9, and GFAP in brain versus blood after HI in murine neonates. Immunoblotting suggested that the presence of multiple GFAP bands and the increase of MMP-9 or OPN are all sensitive markers of HI injury in the brain (Fig. 3A). In contrast, only induction of OPN, but not MMP-9 or GFAP, was clearly detectable in the blood of these HI-injured animals at 24 h recovery (Fig. 3B). Moreover, multiplex ELISA (Luminex) showed strong correlation between plasma OPN levels and OPN (Fig. 3C; p = 0.0038) and MMP-9 (Fig. 3D; p = 0.0115; n = 10) levels in the brain at 24-h recovery. These results suggest that plasma OPN is a monitoring biomarker for brain damage in experimental HI.
To examine the timing of plasma OPN induction after HI, we collected serial blood samples at 6, 12, 24, 48, 72, and 96 h postinjury from a cohort of postnatal day 7 (P7) rats for immunoblotting. This analysis showed variable dynamics of OPN alterations in HI-injured animals, but strong induction (>5-fold) occurred only 24 h post-HI (Fig. 3E; n = 5). These data suggest that the increase of plasma OPN may indicate the minimal onset time of perinatal asphyxia.
Finally, to test the prognostic value of plasma OPN for HI brain damage, we shortened the duration of hypoxia (to 35 min) to produce more variable brain damage in the same cohort of mouse pups \((n = 11)\) and examined the relationship between the severity of brain injury at 7 d post-HI and the corresponding plasma OPN levels at 48 h (Fig. 3F). We found that mice with mild brain injury \((n = 5)\) exhibited low plasma OPN levels that were indistinguishable from baseline at 48 h post-HI, whereas animals with severe brain damage \((n = 6)\) showed higher plasma OPN levels than baseline at 48 h post-HI (Fig. 3G). The 48 h plasma OPN levels between mice that would later develop mild- or severe- brain damage were not significantly different, however, owing to outliers in each group. These results suggest that plasma OPN levels have predictive value for the severity of HIE brain injury, but multiple time-point monitoring or the use of additional biomarkers is needed to increase prognostic accuracy.

**Discussion**

The objective of this study is to test the applicability of quantitative proteomic methods toward the discovery of blood biomarkers for HI brain injury. Although therapeutic hypothermia (to 32–34°C) starting within 6 h after HI insult and continuing for 12–72 h improves outcomes, 40%–50% of infants treated with hypothermia still die or develop significant neurological disabilities. Thus, there is a need for better HIE therapies and reliable biomarkers to monitor response to treatment (Bennet et al., 2010). A recent study indicated that magnetic resonance imaging performed at 44 weeks postmenstrual age or when clinically feasible is a reliable indicator of neurodevelopmental outcomes at 6–7 y of age (Shankaran et al., 2015). Yet simple laboratory assays using blood or urine that can be performed more widely and acutely remain a highly useful tool in clinical care of HIE. To this end, past research using a candidate approach has suggested several blood biomarkers in HIE, such as GFAP and MMP-9 (Bembea et al., 2011; Bednarek et al., 2012). In contrast, an open-ended proteomic strategy has not been used, despite its application in identifying blood biomarkers in obstetrical conditions (Klein et al., 2014). In preclinical study, only 2D differential gel electrophoresis has been used to compare brain proteins after HI (Rosenkranz et al., 2012). To our knowledge, the present study is the first report using quantitative proteomics to identify blood biomarkers in experimental HI. Our results support the use of quantitative proteomics for biomarker discovery and suggest osteopontin as a prognostic blood biomarker in HI brain injury.

In our experimental design, the control group of low-dose i.p. LPS exposure (which does not produce obvious brain damage) is designed to avoid non–lesion-associated immune alterations in LPS/Hi injury. Using MS and statistical analysis of the plasma proteins in four experimental groups, we revealed 16 proteins that are associated with both HI and LPS/Hi brain injury. Among them, we selected OPN and cystatin C for validation, given their relevance to neonatal asphyxia in the literature, and saved the other candidates for future study. Specifically, the plasma cystatin C level has been suggested to be a biomarker of renal injury in neonatal asphyxia (Allegaert et al., 2015). Induction of protein and mRNA of OPN was also reported in HI-injured rodent neonatal brains (Chen et al., 2011; van Velthoven et al., 2011).

### Table 1. Plasma proteins that are uniquely associated with HI and LPS/HI brain injury at 24-h recovery

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description (all in <em>Mus musculus</em>)</th>
<th>Peptide feature counts</th>
<th>CT/CT SD (ratio)</th>
<th>CT/CT log2</th>
<th>LPS/CT SD (ratio)</th>
<th>LPS/CT log2</th>
<th>HI/CT SD (ratio)</th>
<th>HI/CT log2</th>
<th>LPS/Hi/CT SD (ratio)</th>
<th>LPS/Hi/CT log2</th>
<th>LPS/Hi/CT SD (ratio)</th>
<th>LPS/Hi/CT log2</th>
</tr>
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<tbody>
<tr>
<td>TUBA1B</td>
<td>Tubulin alpha-1B chain</td>
<td>58</td>
<td>-0.6 0.33</td>
<td>-0.78 0.5</td>
<td>-0.94 0.47</td>
<td>-1.11 0.41</td>
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<td></td>
</tr>
<tr>
<td>RELN</td>
<td>Reelin precursor</td>
<td>18</td>
<td>0.01 0.22</td>
<td>-0.57 0.35</td>
<td>-0.96 0.27</td>
<td>-1.05 0.14</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MBL2</td>
<td>Mannos-binding protein C precursor</td>
<td>86</td>
<td>0.23 0.28</td>
<td>-0.51 0.43</td>
<td>-1.17 0.3</td>
<td>-1.55 0.3</td>
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<tr>
<td>TAS1R3</td>
<td>Taste receptor type 1 member 3 precursor</td>
<td>5</td>
<td>-0.35 0.17</td>
<td>-0.77 0.43</td>
<td>-1.22 0.53</td>
<td>-1.38 0.56</td>
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<tr>
<td>IGFALS</td>
<td>Insulin-like growth factor-binding protein complex acid labile subunit precursor</td>
<td>171</td>
<td>-0.11 0.26</td>
<td>-0.56 0.18</td>
<td>-1.78 0.34</td>
<td>-2.06 0.31</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IGFBP3</td>
<td>Insulin-like growth factor-binding protein 3 precursor</td>
<td>27</td>
<td>-0.19 0.16</td>
<td>0.13 0.16</td>
<td>-0.92 0.25</td>
<td>-1.28 0.35</td>
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<td></td>
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<tr>
<td>GDA</td>
<td>Guanine deaminase</td>
<td>15</td>
<td>0.25 0.36</td>
<td>0.62 0.34</td>
<td>1.03 0.45</td>
<td>1.05 0.49</td>
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<td></td>
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<tr>
<td>OSTB</td>
<td>Organic solute transporter subunit beta</td>
<td>8</td>
<td>0.42 0.14</td>
<td>0.79 0.29</td>
<td>0.9 0.31</td>
<td>0.15 0.05</td>
<td></td>
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<tr>
<td>OPN</td>
<td>Osteopontin</td>
<td>11</td>
<td>0.12 0.4</td>
<td>0.69 0.28</td>
<td>1.68 0.6</td>
<td>1.31 0.43</td>
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<tr>
<td>STFA3</td>
<td>Stefin-3 (cystatin C)</td>
<td>14</td>
<td>-0.48 0.99</td>
<td>0.62 0.68</td>
<td>2.63 0.71</td>
<td>1.67 0.6</td>
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<tr>
<td>MY018B</td>
<td>Myosin XVIIIb</td>
<td>2</td>
<td>-0.25 0.15</td>
<td>0.8 0.25</td>
<td>1.51 0.31</td>
<td>1.3 0.2</td>
<td></td>
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</tr>
<tr>
<td>SH3BGLR</td>
<td>SH3 domain-binding glutamic acid-rich like protein</td>
<td>3</td>
<td>0.33 1.21</td>
<td>-0.09 1.98</td>
<td>1.86 0.84</td>
<td>1.7 0.76</td>
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<tr>
<td>DCPS</td>
<td>m7GppX diphosphatase</td>
<td>12</td>
<td>0.2 0.35</td>
<td>0.34 0.45</td>
<td>1.55 0.61</td>
<td>1.47 0.32</td>
<td></td>
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</tr>
<tr>
<td>PLEKHM1</td>
<td>Pleckstrin homology domain-containing family M member 1</td>
<td>2</td>
<td>-0.05 0.49</td>
<td>-0.12 0.45</td>
<td>0.92 0.42</td>
<td>1.06 0.44</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CYCS</td>
<td>Cytochrome c, somatic</td>
<td>50</td>
<td>-0.35 0.14</td>
<td>0.64 0.21</td>
<td>1.59 0.23</td>
<td>1.44 0.28</td>
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<tr>
<td>SCARB1</td>
<td>Scavenger receptor class B member 1 isoform 1</td>
<td>2</td>
<td>-0.25 0.1</td>
<td>0.71 0.25</td>
<td>1.3 0.4</td>
<td>1.75 0.46</td>
<td></td>
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Plasma proteins associated with HI and LPS/HI brain injury at 24-h recovery in P10 mice. Proteomics detection and statistical analysis were performed as described in the text. Sixteen high-confidence proteins changing in this category with \( \geq 2 \) MS/MS-identified peptide feature counts used for quantitation across the 12 samples are reported.
Our immunoblot results validated the increase of both OPN and Cystatin C in blood after HI and LPS/HI injury, but we decided to further characterize OPN given its low basal level (which assists the detection of postinjury induction) and lesion-side specific induction in HI- and LPS/HI-injured brains. These characteristics raise the possibility that post-HI plasma OPN may derive from the injured brain, a scenario that is further supported by two observations. First, we showed that HI induces Opn mRNA in brain, but not in blood, consistent with a previ-

Figure 2. Source and specificity of plasma OPN as a biomarker. **A**, RT-PCR showed that HI triggered a 20- to 30-fold increase of Opn mRNA in the brain of P10 mice and P7 rats at 24-h recovery, but only insignificant changes in PBMCs (n = 4–5 for each group). **B**, Immunolabeling showed widespread OPN expression by Iba1⁺ microglia and occasional expression in NG2⁺ glial progenitors, but rare expression in GFAP⁺ astrocytes after HI injury (n > 4). Scale bar: 40 μm. **C**, Application of LPS (1 ng/ml) to microglial SM826 cells in vitro led to induction of OPN protein. **D**, Similarly, ICV injection of LPS (1 μg) induced Opn mRNA in brain as well as plasma OPN protein at 24-h recovery.
ous finding of brain-specific induction of Opn mRNAs in cerebral ischemia models (Tang et al., 2002, 2003). Second, cerebral ventricular injection of LPS, a model of selective microglia activation, was able to elevate plasma OPN levels, whereas i.p. injection of 10-fold, 3-mg/kg LPS, a model to induce systemic immune responses, lacked this effect.

In addition, our results suggest that plasma OPN is more sensitive than MMP-9 and GFAP as a biomarker of experimental HI brain injury. The higher sensitivity of OPN after HI injury may relate to its intrinsic property as a secreted protein or its greater stability in blood (Lanteri et al., 2012). We also showed that plasma OPN induction typically occurs after 24 h of HI onset and closely correlates with brain MMP-9 levels, and that high levels of plasma OPN at 48 h post-HI correlate with severe brain injury at 7 d. Whether human HIE neonates show similar induction of plasma OPN warrants further investigation. If a similar pattern is observed, monitoring plasma OPN levels in neonates may serve two purposes. First, an
increase of plasma OPN may indicate the minimal onset time of perinatal HI (~24 h), which would suggest intravascular HI stress and poorer response to hypothermia treatment. Second, the trajectory of plasma OPN alterations may indicate the progression or recovery of brain injury in postasphyxia neonates. Importantly, our results do not exclude the possibility that plasma OPN may also derive from activated macrophages outside the brain. Hence, differential diagnosis and multiple-tissue monitoring remain essential for the interpretation of plasma OPN induction in neonatal care.

MS-based proteomics is a powerful strategy to determine disease-associated biomarkers in biofluids, including blood, urine, and cerebrospinal fluid. With this methodology, no additional *a priori* hypothesis of the biomarker is needed, and once a protein biomarker is found, simple and relatively cheap laboratory tests can be developed for multiple time-point monitoring of disease progression, even in less-developed areas of the world. Moreover, when applied to well-controlled samples, quantitative proteomics is a very efficient method to uncover differential biomarkers. For example, the present study used only four experimental groups and three mice per group, but we were able to uncover OPN as a blood biomarker of HI brain injury by statistical analysis. By adding comparison groups and enlarging the sample size, this quantitative proteomic strategy may reveal additional blood biomarkers in HIE.

In summary, our findings support the application of proteomic analysis to identify biomarkers in HIE. Moreover, the prognostic value of plasma OPN in HI brain injury warrants investigation in human infants.

**References**


