Elucidating novel mechanisms of brain injury following subarachnoid hemorrhage: an emerging role for neuroproteomics

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Subarachnoid hemorrhage (SAH) is a devastating neurological injury associated with significant patient morbidity and death. Since the first demonstration of cerebral vasospasm nearly 60 years ago, the preponderance of research has focused on strategies to limit arterial narrowing and delayed cerebral ischemia following SAH. However, recent clinical and preclinical data indicate a functional dissociation between cerebral vasospasm and neurological outcome, signaling the need for a paradigm shift in the study of brain injury following SAH. Early brain injury may contribute to poor outcome and early death following SAH. However, elucidation of the complex cellular mechanisms underlying early brain injury remains a major challenge. The advent of modern neuroproteomics has rapidly advanced scientific discovery by allowing proteome-wide screening in an objective, nonbiased manner, providing novel mechanisms of brain physiology and injury. In the context of neurosurgery, proteomic analysis of patient-derived CSF will permit the identification of biomarkers and/or novel drug targets that may not be intuitively linked with any particular disease. In the present report, the authors discuss the utility of neuroproteomics with a focus on the roles for this technology in understanding SAH. The authors also provide data from our laboratory that identifies high-mobility group box protein-1 as a potential biomarker of neurological outcome following SAH in humans. (DOI: 10.3171/2009.10.FOCUS09223)

KEY WORDS • early brain injury • hemorrhagic stroke •
cerebral aneurysm • inflammation • cerebrospinal fluid • biomarker

NEUROLOGICAL injuries are associated with long-term disability and significant patient death. Aside from a significant emotional toll, brain injuries place a massive economic burden on society each year. Despite decades of intense investigation, clinical treatment options remain limited, in part, due to the poorly defined sequelae underlying injury progression. The cellular pathways culminating in neurological demise are likely activated within the first minutes to hours following the injury, suggesting that early diagnosis and intervention may be paramount to improving patient prognosis.

The identification of clinically viable therapeutics to limit brain injury remains a subject of intense research focus. Over the past several decades, preclinical research proceeded in a logical, hypothesis-driven manner in which the involvement of a single gene or protein was tested for a given biological function based on existing

reports in the literature. Unfortunately, despite great promise in preclinical trials, species differences, drug delivery issues, and poor brain penetration contributed to the inability of most experimental drugs to significantly improve patient outcomes following brain injury. Thus, the incorporation of innovative research strategies may be required to further elucidate the mechanisms of brain injury at the molecular and cellular levels and provide novel targets for improved drug design.

In contrast to the systematic and laborious task of investigating the role of 1 established gene at a time, the advent of genomic and proteomic approaches allows the simultaneous, large-scale screening of all gene/proteins in a biological sample. These advanced screening techniques also allow objective, nonbiased data collection, permitting the identification of biomarkers and/or novel drug targets that may not be intuitively linked with any disease process. These findings may then be exploited in further preclinical and clinical testing. Although these technologies are often criticized as nonhypothesis driven, genomic and proteomic screening methods have significantly increased the mechanistic understanding of

Abbreviations used in this paper: BBB = blood-brain barrier; HMGB = high-mobility group box protein; IL = interleukin; NPH = normal-pressure hydrocephalus; NVU = neurovascular unit; SAH = subarachnoid hemorrhage; TLR = toll-like receptor.

numerous physiological and pathological processes and aided in the identification of disease biomarkers.

We believe that the field of neurosurgery stands to greatly benefit from these rapidly evolving technologies, both in the diagnosis (biomarker discovery) and therapeutic intervention (target discovery, validation, and novel drug design) of complex neurovascular pathologies. To maintain focus, the potential applicability of advanced genomics/proteomics will be discussed in the context of SAH, a type of hemorrhagic stroke caused by the spontaneous rupture of a cerebral aneurysm. Original data from our research group, demonstrating the utility of proteomic screening of patient specimens in novel drug target and biomarker identification, will also be provided.

Therapeutic Targeting Following SAH: Many Questions, Few Answers

Subarachnoid hemorrhage remains a major cause of death and disability in the US, with a prevalence of 1 in 10,000 people (approximately 7% of all strokes).^{29,61} Although the overall incidence of stroke declined over the past several decades, the frequency of SAH remains stable despite medical advancements.¹¹¹ Patients with SAH exhibit a 30-day mortality rate of 30–40% and sustain a loss of productive life years comparable to that of patients with cerebral infarction, due in part to the young age of onset, dearth of viable therapeutic options, and poor clinical prognosis.^{29,38,62,64} The estimated lifetime cost per SAH patient is double that of a patient with ischemic stroke, as ~ 50% of patients with SAH experience permanent disability (such as deficits in verbal and nonverbal memory, psychomotor speed, executive function, and visual-spatial function).^{21,39,45,56,64} Overall, ~ 70% of patients with SAH die or require long-term assisted care due to neurological impairments.

The dogmatic view of SAH suggests that delayed cerebral vasospasm, a progressive narrowing of the large cerebral arteries ~ 4–10 days postictus, is the primary cause of neurological demise and death following SAH. Based on the clinical correlation between the onset of cerebral vasospasm and neurological deterioration, intense research efforts were focused on limiting large artery constriction with the hope that this would improve patient outcome. Unfortunately, a recent clinical trial showed that clazosentan, an endothelin receptor antagonist, successfully reduced angiographic vasospasm by ~ 65% without a corresponding improvement in neurological function or 3-month patient outcome.⁴⁰ Consistent with this finding, preclinical data from our laboratory and others indicated a similar functional dissociation between cerebral vasospasm and neurological outcome.^{74,114} Together, these unexpected findings challenge the view that delayed cerebral vasospasm is solely responsible for brain injury following SAH and suggest that a reevaluation of the disease process may be required.^{40,72,74,84,114} Although a detailed assessment of the state of the field is beyond the scope of this report, the reader is directed to several excellent commentaries on the current challenges associated with managing SAH.^{40,70,84}

Early Brain Injury as a Cause of Subsequent Neurological Demise?

Early brain injury, a group of detrimental neurovascular pathologies that occur in the acute injury phase following SAH, includes cortical spreading depression, neuroinflammation, microvascular injury, BBB opening, and global cerebral edema.^{6,16,21,22,25,26,55,58,68,69,97,101,113,119} Although the initiating events in the cause of early brain injury remain unclear, changes within the NVU are implicated in neurological demise following SAH.^{82,123–125} The NVU is composed of neurons, glia, and microvessels organized into discrete units, each of which may communicate with and influence the physiology of the other cell types. For example, astrocytes are in juxtaposition to both neurons and endothelial cells and functionally influence the formation and maintenance of the BBB,^{1,47,56} regulation of cerebral blood flow in response to neuronal activity,^{46,59,103} maintenance of oxidative balance,⁵⁶ promotion of synaptogenesis, and neuroprotection.^{71,83,108,109,114} Thus, perturbations within the NVU may negatively impact brain function after SAH. Along these lines, a vasoconstrictive response generated within minutes of SAH reduced cerebral blood flow and initiated a damaging cascade of events, including enhanced ischemic brain injury and early death, following experimental and clinical SAH.^{9,12,56,89,91,114,118}

Understanding Neurological Injury After SAH: Where Do We Go From Here?

Whereas novel avenues of exploration into the mechanisms underlying neurological demise (beyond cerebral vasospasm) are clearly needed, the lack of a focused research direction presents a major obstacle in the field. Although there is growing appreciation for the role of early brain injury in determining neurological outcome following SAH, elucidating the complex cellular interactions within the NVU presents unique and technically challenging issues. As such, the traditional research model involving the development of a hypothesis centered upon a single gene/protein in disease progression followed by resource (time, labor, and cost)-intensive experimentation may not provide adequate mechanistic information in a timely manner. Thus, new experimental approaches and tools will be needed to fully understand the pathogenesis of brain injury following SAH. In the following sections, the potential utility of neurogenomics and neuroproteomics to identify and define the molecular and cellular changes within the NVU following SAH are discussed.

The Genomic Revolution

The information gained by the sequencing of the human genome permits the integration of gene expression, gene mutations, epigenetic modifications, and gene polymorphisms (single nucleotide polymorphisms) with biological/functional outcomes, opening an exciting new era in translational research.^{2,56} Microarray (“gene chip”) analyses permit the large-scale, simultaneous comparison of gene expression between 2 or more study populations (such as healthy control patients vs those with disease),

allowing the identification of individual genes or patterns of gene expression, including those not previously linked with a given disease process. This information may provide novel mechanisms of disease phenotype/progression and/or identify correlations between DNA polymorphisms and disease risk, such as the risk of developing a cerebral aneurysm.

Gene expression profiling technology identified 138 differentially expressed genes within the large cerebral arteries following SAH in rats, providing potential mechanisms of delayed cerebral vasospasm and neurological demise.¹¹² Of these changes, a known function was ascribed to 77 genes using the Online Mendelian Inheritance in Man (OMIM) database (<http://www.ncbi.nlm.nih.gov/omim/>), which houses genetic information on human disorders and diseases, including a large number implicated in processes related to inflammation, metabolism, oxidative stress, and regulation of the extracellular matrix. A similar study reported the upregulation of 18 genes associated with inflammation and cellular injury within vasospastic cerebral arteries following SAH in dogs,⁷⁷ suggesting a possible role for inflammatory mediators following the rupture of a cerebral aneurysm.

Inflammation may signal an adaptive response to promote tissue repair,^{23,43} but uncontrolled or chronic inflammation, such as that observed during Alzheimer disease, Parkinson disease, and ischemic brain injury, irreversibly damages tissue and promotes oxidative stress.^{44,60,66,67,76,80} Mechanistic studies to support the functional validity of the preclinical microarray data remain largely unexplored; however, initial clinical observations and preclinical data from our laboratory and others suggest that antiinflammatory compounds attenuate acute brain injury following SAH.^{48,56,73,85,114,116} For example, mice overexpressing the gene for extracellular superoxide dismutase or copper-zinc superoxide dismutase exhibited a reduction in the development of cerebral vasospasm and attenuation of oxidative stress.^{49,72,92} Similarly, CSF or serum from patients with SAH exhibited more immune complexes, complement activation, and increased levels of oxidative and inflammatory mediators (such as IL-6, tumor necrosis factor- α , and intercellular adhesion molecule-1) as compared with control patients.^{20,32,34,42,50,56,87,90,101} These preliminary findings indicate the utility of microarray analysis for advancing the mechanistic understanding of acute brain injury following SAH and provide potential therapeutic targets for future preclinical and clinical studies.

Genomic studies after SAH in humans remain completely unexplored, in part due to the ethical and technical issues associated with the collection of tissue samples from patients with SAH. Whereas preclinical models of hemorrhagic stroke provide abundant access to mRNA, brain tissue from patients with SAH is not readily available. In contrast, blood is readily obtained from patients during routine clinical care, allowing blood genomic expression profiling. Although unreported following SAH, microarray analysis using peripheral blood mononuclear cells collected from patients with cerebral ischemia revealed an increase in the expression of hypoxia-induced stress genes, vascular repair genes, and neuroprotective genes, as compared with blood collected from control pa-

tients. Although these types of studies potentially provide unique clinical insights into the pathophysiology of brain injury, it remains unclear whether blood genomic responses accurately reflect the brain response to injury (for example, whether immune cells exhibit the identical genomic response as neurons following hypoxia-ischemia). Similarly, it is unknown whether the observed changes in peripheral blood cells indicate specific responses to the injury or whether these changes actually reflect other preexisting conditions within individual patients, such as vascular disease, hypertension, and diabetes.^{7,28}

Together these findings suggest that the methods of sample collection and data analysis must be carefully considered when interpreting the findings from these types of studies. Additionally, mRNA expression is dynamic and varies from minute to minute, requiring the ability to repeatedly collect living cells over time. Finally, changes in mRNA expression are a relatively poor predictor of protein expression and biological activity.³⁷ Thus, while genomics provides an important foundation for identifying novel therapeutic targets, these important caveats may diminish the utility of gene expression following SAH in humans. As such, changes in protein expression and/or modification may provide more meaningful information regarding the complex mechanisms underlying neurological injury following SAH. In the following section, we discuss the potential applicability of proteomics in the study of brain injury.

Proteomics: the Next Step on the Journey

The complement of proteins within a cell or tissue (called the proteome) is considerably larger and more complex than the human genome. For example, alternative splicing of a single transcript may result in several different isoforms of a protein, significantly increasing the number and diversity of proteins. Further complicating the analysis of proteins following brain injury, post-translational protein modifications such as phosphorylation, glycosylation, and myristoylation modulate protein activation states and protein-protein interactions, providing additional complexity. Thus, advanced technologies are needed to identify the key mediators of neurovascular injury. Once identified, preclinical modeling studies, such as those that use transgenic mice that lack or overexpress a molecule of interest, may be used to define whether a given protein is protective, detrimental, or both. In this section, we highlight the potential utility of proteomic analyses following brain injury. This is followed by the presentation of data from our laboratory, demonstrating the use of proteomic screening in patient-derived CSF to identify a possible biomarker/cellular mechanism of brain injury following SAH.

Proteomics, a method of emerging clinical importance, permits a direct comparison of protein expression between 2 or more populations (controls vs patients with disease). A major benefit of proteomic research is that novel proteins may be discovered and linked with a disease process for the first time, without the need for an existing hypothesis or precedent for that particular protein in the given disease process. To accomplish this goal,

samples of interest are resolved by 2D gel electrophoresis, a method that separates complex protein mixtures by charge, then by molecular weight,¹⁸ resulting in the generation of a 2D gel electrophoresis map and a reference map of all proteins in a given sample. By comparing the spot location for an individual protein between 2 populations, changes in protein expression may be determined. Spots that show distinct differences between experimental groups can then be analyzed by techniques such as liquid chromatography-tandem mass spectrometry, which combines 1 or more chromatographic steps with 2 rounds of mass spectrometry.⁷⁴ This procedure allows the identification of individual proteins within complex, heterogeneous biological samples, including serum and CSF.

Proteomic Subdivisions

Bayés and Grant⁸ classified proteomics into 4 distinct subdivisions. The first subdivision, expression neuroproteomics, involves the qualitative and quantitative profiling of the proteome and is traditionally accomplished using gel electrophoresis.⁷⁸ The second subdivision, functional neuroproteomics, studies the functional properties of individual proteins, including posttranslational modifications and organization of proteins into substructures, complexes, and networks. Data obtained using this method has improved the understanding of complex biological systems, such as the molecular organization of postsynaptic density, which would not otherwise be possible with genomic analyses.⁹⁴ For example, many proteins involved in the presynaptic apparatus^{14,102} and the postsynaptic anchoring and clustering of *N*-methyl-D-aspartate-type glutamate receptors were identified using this method.^{27,33,52,59,63,75}

Clinical neuroproteomics, the third subdivision, focuses on drug discovery and on the identification of novel biomarkers and disease mechanisms for neurological, neurodegenerative, and psychiatric diseases.^{17,41,105,110,115,117} In particular, CSF provides an increasingly important resource for identifying novel changes within the brain. The final subdivision, neuroproteomic informatics, addresses the computation tools and databases necessary for handling and analyzing complex proteomic data sets. These technologies are important for determining statistically meaningful data and for the establishment of databases and repositories for proteomic data, which may be mined at a later date by other investigators. In the context of neurosurgery, we believe that clinical neuroproteomics may be particularly useful for identifying novel biomarkers of disease (in controls vs patients with disease) and/or providing novel cellular targets for future drug discovery. As such, the remainder of this review will focus on the potential applicability of this subcategory of neuroproteomics following SAH.

Cerebrospinal Fluid: A Gateway to the Brain?

Human brain specimens are not readily obtainable from patients with SAH, but CSF diversion is routinely performed in the neurointensive care unit, providing an easily accessible source of proteins from patients. Cerebrospinal fluid circulates throughout the brain and con-

tains high amounts of protein (~ 15–40 mg/dl),³⁰ making this the sample of choice for novel biomarker discovery using proteomic methodologies. Proteomic screening of CSF was first performed nearly 4 decades ago to provide novel insights into human brain physiology and disease.^{24,31,51} Initial studies performed in 1980 using 2D gel electrophoresis revealed ~ 300 proteins in human CSF, although most of these proteins remained unidentified at this time.³⁵ Subsequent studies in the early 1990s, using improved technologies, detected ~ 1000 proteins of which 248 were identified.¹²¹ More recent studies in 2007 using liquid chromatography-tandem mass spectrometry revealed the presence of ~ 2500 proteins,^{80,81} providing essential information to generate an atlas of the human CSF proteome, a valuable resource that provides a potential source of brain disease biomarkers.⁸¹ Given the availability of patient CSF within an academic medical center, our research group attempted to identify novel biomarkers of neurological injury following SAH, which may aid in the early diagnosis and treatment of these patients. These original data are presented in the following section.

High-Mobility Group Box Protein-1: A Predictive Biomarker of Neurological Outcome Following SAH?

Using proteomic screening technologies, we detected the expression of a 25 kD protein, HMGB1, in the CSF of all 9 of the patients with SAH who we analyzed. In contrast, HMGB1 was below the level of detection in the CSF of all 7 control patients (Fig. 1), suggesting HMGB1 release may be specific to brain injury. Notably, the levels of HMGB1 within the CSF retrospectively correlated ($r = 0.786$) with neurological outcome, as determined by the Hunt and Hess grading scale (Fig. 2A), and were highly correlated ($r = 0.938$) with the degree of disability or dependence at patient follow-up examinations, as assessed by the modified Rankin scale (Fig. 2C). In contrast, the HMGB1 content of CSF was not strongly correlated ($r = 0.334$) with the appearance of SAH on CT scans, as determined by the Fisher grade (Fig. 2B). Together, these novel data implicate HMGB1 as a possible biomarker for neurological injury and as a predictive marker of patient outcome following SAH. These findings also provide a rationale for characterizing the functional role of HMGB1 in promoting brain injury after SAH.

High-mobility group box protein-1, also called HMG or amphoterin, is an evolutionarily conserved, nonhistone DNA binding protein that is constitutively expressed in most cells throughout the body, including the brain.^{57,106} Under physiological conditions, HMGB1 localizes to the nucleus to stabilize nucleosomal structure and to facilitate gene transcription.¹⁵ In contrast, HMGB1 functions as a proinflammatory cytokine when translocated into the extracellular space.^{3,4,19,36,56,105} Thus, HMGB1 may be classified as an “alarmin,” a multifunctional host protein that activates an immune response to warn neighboring cells of injury.^{10,99} Consistent with this assertion, HMGB1 is increased in the CSF in patients with meningitis¹⁰⁵ and in the serum of patients with cerebral ischemia,³⁶ suggesting HMGB1 may represent a marker of neurological injury.

Fig. 1. A: Representative Western blot of HMGB1 in CSF samples collected from patients with aneurysmal SAH (lanes 1–5) and a control patient with NPH (lane 6). Blots were imaged using a Li-Cor Odyssey near-infrared imaging system. Patients with SAH consistently presented with an ~ 25 kD band corresponding to HMGB1, whereas patients with NPH did not show a clear band. **B:** Noncontrast axial CT scans of a 45-year-old woman with a Hunt and Hess grade of III and a Fisher grade of 4. The patient was sleepy and confused following a ruptured left posterior communicating artery aneurysm. The patient's HMGB1 sample is depicted in lane 2 (A). **C:** Noncontrast axial CT scans of a 77-year-old woman with a Hunt and Hess grade of IV and a Fisher grade of 4. The patient was stuporous with significant right hemiparesis following a ruptured left middle cerebral aneurysm. The patient's HMGB1 sample is depicted in lane 5 (A).

The function or functions of HMGB1 following SAH remain completely unexplored; however, intracerebroventricular administration of HMGB1 induced the expression of proinflammatory mediators (such as IL-1 β , tumor necrosis factor- α , and IL-6) within the rodent brain.^{3,76} The mechanisms whereby extracellular HMGB1 increases the expression of neuroinflammatory mediators remain unstudied, but activation of the receptor for advanced glycation end products, TLR2, and/or TLR4 mediate the effects of HMGB1 in the periphery.^{5,53,54,56,77,82,100,107,122} Along these same lines, HMGB1 activated the proinflammatory transcription factor nuclear factor- κ B via a TLR-dependent pathway in mouse macrophages and human kidney cells.⁸² Consistent with these reports, the acute expression of TLR4 within astrocytes and vascular endothelium correlated with increased nuclear factor- κ B activation and neuroinflammation following SAH in rats.^{68,69} Because neuroinflammation is an important component of early brain injury and neurological demise after SAH,^{82,86,87,96,98,101,104,114,120} HMGB1 may represent a clinically relevant, mechanistic link between acute injury and secondary neurovascular injury following SAH.

In response to inflammatory mediators, such as those that are increased after SAH, macrophages,^{11,19} natural killer cells,⁹⁵ and myeloid dendritic cells⁶⁵ actively secrete HMGB1. In this case, the detection of elevated HMGB1 levels within the CSF may signal an adaptive immune response to clear cellular debris and promote resolution

following a cerebral hemorrhage. In contrast, HMGB1 is not actively secreted within the CNS, but it is passively released into the extracellular space and CSF by necrotic neurons following cerebral ischemia.⁸⁸ Consistent with these findings, recent data from our laboratory suggests that neuronal HMGB1 is specifically released within the cerebral cortex following hemorrhagic stroke in mice (C.H.A. and K.M.D., unpublished observation, 2009). Thus, neuronally derived HMGB1 may provide an early marker of neurological injury, which if therapeutically targeted, could attenuate early brain injury after SAH. Ongoing work in our laboratory is actively characterizing the contributions of both immune and neuronal cells toward the post-SAH release of HMGB1. This knowledge will undoubtedly improve our understanding of disease pathophysiology and may provide a marker of acute neuronal injury after SAH (and possibly other neurological injuries).

Future Prospects, Challenges, and Direction of the Field

Despite significant advances in neurosurgical approaches, improvements in patient diagnosis, and intense research efforts, the mortality and morbidity following SAH remain unchanged.⁹³ The notion that neurological demise following SAH is solely caused by the development of delayed cerebral vasospasm was challenged by a number of recent preclinical and clinical reports, in-

Fig. 2. Graphs of the retrospective correlation analysis of HMGB1 content within the CSF of patients with SAH according to the Hunt and Hess grade (**A**), Fisher grade (**B**), and modified Rankin scale score (**C**) at follow-up examination. High-mobility group box protein-1 was quantified by Western blotting. All grades were provided by the attending physician, who was blinded to experimental studies. High-mobility group box protein-1 was not present in the CSF of control patients with NPH.

cluding work by our laboratory,^{40,74,114} emphasizing the need for novel therapeutic targets and treatment modalities. Early brain injury is a primary cause of death in patients with SAH^{9,13} and emerged as a possible therapeutic target following SAH. Ongoing research suggests early brain injury involves complex neurovascular pathologies, including delayed cerebral ischemia, global edema, BBB disruption, cortical spreading depression, and neuroinflammation;^{16,79} however, the signaling pathways and mechanisms involved in the initiation of these events re-

main largely unknown. Thus, innovative new approaches may be required to understand these complex processes.

Genomic and proteomic analyses are rapidly emerging as important tools for deciphering the complex neurovascular interactions in human physiology and disease. In particular, the incorporation of proteomics into the study of neurological diseases such as SAH may provide exciting new insights into the cellular mechanisms of brain injury. Unlike the retrospective study of individual genes/proteins in postmortem patient specimens, neuroproteomics: 1) allows a sensitive and unbiased method to identify novel proteins associated with a given disease process (biomarkers); 2) can be used to identify novel proteins without the need for a specific hypothesis prior to experimentation; 3) is useful for analyzing patient samples, such as CSF and serum, which are collected in the routine care and treatment of patients on the neurosurgical unit; 4) can aid in deciphering complex cellular interactions, subcellular networks (synaptic function), and posttranslational modifications; 5) can support the development of large databases allowing the comparison of data between multiple medical centers; and 6) can be performed using equipment that is available at most major medical centers. Together, we believe these features of neuroproteomics will allow the widespread incorporation of this technology into clinical research and will provide important new insights regarding the mechanisms underlying SAH and other brain injuries.

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