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Multiplexed protein profiling after aneurysmal subarachnoid hemorrhage: Characterization of differential expression patterns in cerebral vasospasm

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Abstract

Cerebral vasospasm is major contributor to delayed morbidity following aneurysmal subarachnoid hemorrhage. We sought to evaluate differential plasma protein levels across time in patients with aneurysmal subarachnoid hemorrhage to identify potential biomarkers and to better understand the pathogenesis of cerebral vasospasm. Nine female patients with aneurysmal subarachnoid hemorrhage underwent serial analysis of 239 different serum protein levels using quantitative, multiplexed immunoassays (DiscoveryMAP 250+ v2.0, Mryiad RBM, Austin, TX USA) on post-hemorrhage days 0 and 5. A repeated measures analysis of variance determined that mean protein concentration decreased significantly in patients who developed vasospasm *versus* those who did not for alpha-2-macroglobulin (F [1.00, 7.00] = 16.33, $p = 0.005$), angiogenin (F [1.00, 7.00] = 7.65, $p = 0.028$), apolipoprotein A-IV (F [1.00, 7.00] = 6.308, $p = 0.040$), granulocyte colony-stimulating factor (F [1.00, 7.00] = 9.08, $p = 0.020$), macrophage-stimulating protein (F [1.00, 7.00] = 24.21, $p = 0.002$), tetranectin (F [1.00, 7.00] = 5.46, $p < 0.039$), vascular endothelial growth factor receptor 3 (F [1.00, 7.00] = 6.94, $p = 0.034$), and significantly increased for vitronectin (F [1.00, 7.00] = 5.79, $p = 0.047$). These biomarkers may be of value in detecting cerebral vasospasm, possibly aiding in the identification of patients at high-risk prior to neurological deterioration.

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Conflicts of Interest/Disclosures

The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

Keywords

Aneurysm; Biomarker; Subarachnoid hemorrhage; Vasospasm

1. Introduction

Aneurysmal subarachnoid hemorrhage is associated with a high mortality rate, often attributed to the initial hemorrhage event or re-hemorrhage prior to aneurysm obliteration. For those who survive these “early events”, cerebral vasospasm can result in impaired cerebral blood flow and is the major cause of delayed ischemia and stroke.¹ Currently available means of detection rely on non-invasive imaging, either with ultrasound or CT angiography. Perfusion based imaging modalities have also been shown to be useful detecting clinically significant alterations in cerebral blood flow. Additionally, catheter based digital subtraction angiography can be used to diagnose vasospasm and is considered the “gold standard”. The purpose of any imaging surveillance for vasospasm is to identify changes in vessel caliber and/or blood flow prior to neurological deterioration. Knowledge of these changes allows for the rapid institution of medical therapies, including systemic hypertension and intra-arterial vasodilator therapy, if patients go on to become symptomatic, in order to prevent permanent deficits.²

While all patients with ruptured cerebral aneurysms have subarachnoid hemorrhage, not all patients go on to develop cerebral vasospasm. Various radiographic grading scales have been developed to demonstrate a relationship between quantity or pattern of blood and the risk of future vasospasm development; however, patients with similar hemorrhages may have a drastically different clinical course, with some patients experiencing no vasospasm at all. This suggests that in addition to the subarachnoid hemorrhage pattern itself, there are patient-specific factors that predispose an individual to cerebral vasospasm. We sought to identify potential systemic biomarkers for cerebral vasospasm following subarachnoid hemorrhage by serial quantitative analysis of serum protein expression profiles spanning dozens of biochemical pathways in patients with ruptured aneurysms.

2. Materials and methods

2.1. Inclusion and exclusion criteria

Following Institutional Review Board approval (MGH – Protocol #2011P002879), potential patients with diffuse aneurysmal subarachnoid hemorrhage were screened for study inclusion over the 10 month study period from November 2012 to September 2013 at a quaternary care, university hospital. Criteria for participation included females >18 years of age with a known time of aneurysm hemorrhage (defined by time of sudden headache onset). Patients who had significant coexisting conditions that might influence baseline serum protein levels (cancer, chronic inflammatory diseases, autoimmune diseases, epilepsy, diabetes, current unstable cardiovascular illness, surgery within the prior 2 weeks, acute infection, and pregnancy) were excluded. Only patients whose aneurysms were treated with microsurgical techniques (craniotomy) were included for participation. Patients needed to have a reasonable chance of survival (subjectively determined by the treating neurosurgeon,

C.S.O.). Written consent was obtained from patients or their surrogates. Patients who had onset of symptoms >24 hours prior to admission were excluded. Any patient enrolled in an interventional clinical trial was excluded.

2.2. Variable and outcome selection

All historical, clinical, radiographic, and follow-up information was collected prospectively. The following data were collected: age, aneurysm location and size, Hunt and Hess grade³, modified Fisher grade⁴, Barrow Neurological Institute grade⁵, maximum transcranial Doppler (TCD) velocity in any vessel, maximum Lindegaard ratio⁶, presence of vasospasm on neuroimaging, development of symptomatic vasospasm, and any endovascular intervention performed for vasospasm.

2.3. Subarachnoid hemorrhage management

All aneurysms were secured within 24 hours of presentation and all patients were managed in a dedicated neurosciences intensive care unit according to a standardized subarachnoid hemorrhage protocol. Phenytoin or levetiracetam was administered until the aneurysm was secured and continuous electroencephalography demonstrated no seizure activity. All patients received oral nimodipine for prevention of delayed ischemic deficits and underwent daily TCD ultrasonography for vasospasm surveillance. CT angiography was performed on patients with elevated or rising TCD values or with a change in neurological status thought to be attributed to vasospasm. Catheter angiography with or without pharmacologic or mechanical intra-arterial vasodilation was performed on patients with CT angiographic vasospasm or on patients in whom there was a high clinical suspicion for vasospasm despite a negative CT angiogram. All patients with vasospasm received systemic hypertensive therapy prior to initiation of endovascular intervention.

2.4. Vasospasm

Vasospasm was defined as new intracranial arterial narrowing (mild, moderate, or severe) identified on catheter or CT angiography (Fig. 1). Elevated TCD velocities of >200 cm/second not thought to be a result of hyperemia were also classified as cases of vasospasm. Symptomatic vasospasm was defined as neurological symptoms referable to a region of radiographic or sonographic vasospasm in the absence of alternative explanations.

2.5. Specimen collection and analysis

Ten ml of peripheral blood was collected from patients within 24 hours of the initial hemorrhage (post-hemorrhage day 0) and again on post-hemorrhage day 5 (two specimen collections total). An analysis of 239 different serum protein levels using quantitative, multiplexed immunoassays (DiscoveryMAP 250+ v2.0, Mryiad RBM, Austin, TX, USA) was performed on each sample. A complete list of analytes is included as supplementary data (Supp. Table 1).

2.6. Statistical analysis

A repeated measures analysis of variance with a Greenhouse–Geisser correction was used to compare samples, using each patient's own baseline sample collected on post-hemorrhage

day 0 as a control. All analyses were performed using the Statistical Package for the Social Sciences version 21 (SPSS Inc., Chicago, IL, USA).

3. Results

Nine patients were enrolled and matched serum samples were collected on post-hemorrhage days 0 and 5. Patient characteristics were collected and are reported in Table 1. Four of the nine patients enrolled developed cerebral vasospasm, none of whom experienced permanent neurological deficit. Sequential analysis of serum protein profiles using multiplexed immunoassays (DiscoveryMAP 250+ v2.0) identified significant decreases in alpha-2-macroglobulin ($F [1.00, 7.00] = 16.33, p = 0.005$), angiogenin ($F [1.00, 7.00] = 7.65, p = 0.028$), apolipoprotein A-IV (APOA4; $F [1.00, 7.00] = 6.308, p = 0.040$), granulocyte colony-stimulating factor (G-CSF; $F [1.00, 7.00] = 9.08, p = 0.020$), macrophage-stimulating protein ($F [1.00, 7.00] = 24.21, p = 0.002$), tetranectin ($F [1.00, 7.00] = 5.46, p < 0.039$), and vascular endothelial growth factor receptor 3 (VEGFR-3; $F [1.00, 7.00] = 6.94, p = 0.034$) in patients who developed vasospasm *versus* those who did not. Additionally, there were significantly increased levels of vitronectin ($F [1.00, 7.00] = 5.79, p = 0.047$) in the vasospasm group.

4. Discussion

Despite similar hemorrhage patterns and clinical characteristics, only a certain proportion of patients will go on to develop cerebral vasospasm following aneurysm rupture. This suggests an incompletely understood mechanism, and implicates the potential contribution of patient-specific factors. In this study, serum analysis of circulating biomarkers were assessed by measuring temporal differences in a broad range of proteins over a 5 day period following aneurysm rupture. Among the 239 proteins tested, only eight demonstrated significant differences between the group of patients who developed vasospasm and those who did not. Early identification of vasospasm development, or even increased susceptibility in any given individual, has the potential to have a dramatic impact on clinical management, including the utilization of intensive care resources, surveillance imaging, and the decision to escalate therapeutics. Our results are notable for the identification of several proteins that to our knowledge have not been previously implicated in cerebrovascular pathology.

4.1. Alpha-2-macroglobulin

Alpha-2-macroglobulin is a large (non-immunoglobulin) plasma protein that functions in a non-specific manner to inactivate a variety of proteinases.⁷ In doing so, the protein limits the fibrinolytic cascade by inhibiting plasmin and kallikrein. It also has several non-peptide binding functions, and may function to regulate the biological activity of cytokines during periods of inflammation.⁸ Cytokines, such as tumor necrosis factor-alpha and interleukin-6, are known to bind to alpha-2-macroglobulin, which modifies their biological effect. Once alpha-2-macroglobulin is activated and bound to a cytokine, it is rapidly removed from the circulation by the hepatic alpha-2-macroglobulin receptor. Increased clearance of alpha-2-macroglobulin, in response to elevated levels of inflammatory cytokines associated with cerebral vasospasm^{9,10}, is a possible explanation for our finding of decreased levels in patients who developed vasospasm.

4.2. Angiogenin

Angiogenin, also known as ribonuclease 5, is a small plasma protein (123 amino acids) that is a potent stimulator of angiogenesis. It has well known actions at the endothelial and vascular smooth muscle cell layers, where it promotes new blood vessel growth through endothelial cell proliferation and invasion.¹¹ Increased angiogenin levels have been seen in several neurological diseases, including large ischemic cerebral infarcts¹² and amyotrophic lateral sclerosis.¹³ However, the potential significance of decreased angiogenin levels in cerebral vasospasm has not been explored to our knowledge. *In vitro* experiments have demonstrated that angiogenin stimulates prostacyclin (a potent vasodilator and inhibitor of platelet aggregation) secretion from the vascular endothelial and smooth muscle cells.¹⁴ Could decreased levels of angiogenin be associated with smaller blood vessel caliber? Further work is needed to establish any associated or causal relationship of angiogenin levels with vasospasm.

4.3. APOA4

APOA4 is a plasma protein most commonly thought to play a role in intestinal lipid absorption and chylomicron assembly.^{15,16} Decreased levels in patients with cerebral vasospasm are of undetermined significance, and there is currently no literature supporting an association. However, other apolipoproteins, primarily apolipoprotein E (ApoE), have been implicated in the pathogenesis of cerebral vasospasm.¹⁷ Targeted replacement of ApoE has even been shown to attenuate vasospasm following hemorrhage in experimental settings.¹⁸ Even though levels of ApoE were not significantly different between groups in our cohort, changes in various lipoprotein expression profiles are possible following subarachnoid hemorrhage. More rigorous evaluation of this protein family and its potential association with vasospasm is necessary.

4.4. G-CSF

G-CSF is a circulating glycoprotein that is best known for its role in the production, differentiation, and function of leukocytes.^{19,20} The direct role of G-CSF on the function of any cerebral vessel is unknown, although potential mediation of an inflammatory response is possible in the context of vasospasm.

4.5. Macrophage-stimulating protein

Macrophage-stimulating protein is a plasma protein growth factor that binds to and activates the receptor tyrosine kinase, Recepteur d'Origine Nantais (RON).²¹ Activation of RON has been implicated in cancer development, where it inhibits classic macrophage activation pathways and promotes alternative activation of macrophages.^{22,23} There is also evidence from animal models that macrophage-stimulating protein and its receptor RON are important in attenuating inflammation. Under physiologic circumstances, activation of this receptor has been shown to suppress cell-mediated inflammation through negative regulation of the interferon-gamma pathway.²⁴ Further investigation is necessary to expand on the preliminary work suggesting the potential role of interferon-gamma in vasospasm.^{25,26} As with any perceived aberration in inflammatory cytokines, care must be

taken to dissect systemic causes of inflammation, such as sepsis or neutrally mediated cardiac dysfunction^{27,28}, from those associated directly with vasospasm.

4.6. Tetranectin

Tetranectin is a plasma protein secreted by neutrophils, monocytes, and macrophages, where it is thought to function primarily as a chemotactic agent.²⁹ It also functions to regulate fibrinolysis and proteolytic processes via binding to plasminogen.³⁰ While tetranectin has been implicated as a potential biomarker in conditions ranging from Parkinson's disease³¹ to cancer³², to our knowledge there are no reports of the role of tetranectin in vasospasm.

4.7. VEGFR-3

VEGFR-3 is a receptor tyrosine kinase that has a prominent role in angiogenesis.³³ Its role in vasospasm has not yet been directly investigated, although the phenomenon of vascular proliferation and subsequent vessel wall thickening in the setting of vasospasm has been associated with increased levels of vascular endothelial growth factor.³⁴ The finding of decreased levels of VEGFR-3 is of unclear significance. Continued study with concurrent cerebrospinal fluid biomarker analysis may elucidate pathogenic mechanisms not detectable in the systemic circulation. Since not all patients with aneurysmal subarachnoid hemorrhage have access to cerebrospinal fluid sampling (as in the case of high grade hemorrhage patients with external cerebrospinal fluid drainage devices), serum biomarkers may have greater applicability than cerebrospinal fluid markers in clinical practice.

4.8. Vitronectin

Vitronectin is an abundant glycoprotein found in human plasma, where one of its functions is to inhibit proteolysis initiated by plasminogen activation. It has also been implicated in cell migration and adhesion, critical components of cancer metastasis.³⁵⁻³⁷ With regards to its potential role in vasospasm, vitronectin has been shown to be a key mediator of vascular smooth muscle cell migration to the neointima.³⁸ It is known that dysfunction of these vascular smooth muscle cells is a well known phenomena in the pathogenesis of vasospasm^{39,40}, exposing a potential avenue of further study.

Overall, results of this study should be interpreted in the context of their preliminary nature. Serum biomarkers collected in this cohort of critically ill patients have the potential to be complicated by more than a single disease entity. Concurrent medical conditions such as pneumonia, heart failure, or underlying occult malignancy, could exist and confound interpretation of the results. Additionally, our sample size was relatively small as a result of the cost necessary to perform the quantitative, multiplexed immunoassays. We chose to use this approach because of its quantitative nature and widespread coverage of several biochemical pathways. Future, prospective studies are needed to investigate some of the preliminary findings suggested here.

5. Conclusion

Serum protein profiles are altered following subarachnoid hemorrhage and differ between patients who recover from their hemorrhage and those who go on to develop vasospasm.

Serum biomarkers have the potential to identify patients at risk for vasospasm development, as well as to aid in the diagnosis of vasospasm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Subarachnoid hemorrhage and vasospasm. (A) Initial non-contrast, axial head CT scan demonstrated diffuse subarachnoid hemorrhage and (B) three-dimensional reconstruction of CT angiogram demonstrated an internal carotid artery aneurysm (grey arrow) as the source of the subarachnoid hemorrhage. (C) Four days following presentation, the patient developed motor weakness in the right upper extremity and word finding difficulty. Digital subtraction angiography demonstrated severe vasospasm (red arrow), with narrowing of the left M1 segment of the middle cerebral artery. (D) The patient was treated with intra-arterial nicardipine with resolution of radiographic vasospasm (blue arrow). All neurological symptoms resolved following treatment.

Table 1

Characteristics of patients with aneurysmal subarachnoid hemorrhage

Patient	Age, years	Location, size (mm)	Radiographic /Sonographic vasospasm	Hunt Hess grade	Fischer grade	BNI grade
1	40	Posterior inferior cerebellar artery, 2	yes	3	3	3
2	41	Anterior choroidal artery, 8	No	1	3	2
3	43	Posterior communicating artery, 5	No	1	3	3
4	45	Anterior communicating artery, 3	Yes	2	3	4
5	47	Anterior communicating artery, 3	No	1	3	2
6	49	Anterior choroidal artery, 2	Yes	2	3	4
7	54	Middle cerebral artery, 10	No	1	3	3
8	59	Posterior communicating artery, 7	No	2	4	4
9	61	Posterior communicating artery, 4	Yes	1	3	4

BNI = Barrow Neurological Institute.