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Disruption of thrombo-inflammatory response and activation of a distinct cytokine cluster after subarachnoid hemorrhage

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ABSTRACT

Background: Unregulated inflammatory and thrombotic responses have been proposed to be important causes of early brain injury and worse clinical outcomes after subarachnoid hemorrhage (SAH).
Objective: We hypothesize that SAH is characterized by an increased inflammatory and thrombotic state and disruption of associations between these states.
Methods: This is a retrospective cohort study of 60 patients with SAH. 23 patients with unruptured aneurysms (UA) and 77 patients with traumatic brain injury (TBI) were chosen as controls. Plasma cytokine levels were measured using a 41-plex human immunoassay kit, and cytokine patterns associated with SAH, UA and TBI were identified using statistical and informatics methods.
Results: SAH was characterized by an increase in several cytokines and chemokines, platelet-derived factors, and growth factors. Cluster analysis identified several cytokine clusters common in SAH, UA and TBI groups – generally grouped as platelet-derived, vascular and pro-inflammatory clusters. In the UA group, the platelet-derived cluster had an inverse relationship with the inflammatory cluster which was absent in SAH. Additionally, a cluster comprising of growth and colony stimulating factors was unique to SAH.
Conclusions: A cluster of cytokines involved in growth and colony stimulation was unique to SAH. Negative associations between the thrombotic and inflammatory molecules were observed in UA but not in SAH. Further studies to examine the pathophysiology behind the cluster unique to SAH and the associations between the thrombotic and inflammatory cytokines are required.

1. Introduction

Subarachnoid hemorrhage (SAH) caused by rupture of a cerebral aneurysm is associated with significant morbidity and mortality [1]. Although the mechanisms of brain injury after aneurysm rupture have been extensively studied, they are still not well understood. Early brain injury (EBI), defined as injury processes occurring within 72 h of aneurysm rupture, exacerbates injury and contributes to poor clinical outcomes [2–4]. Characterizing the pathophysiology during EBI is vital to the development of targeted pharmacological therapies that can mitigate injury expansion and improve outcomes.

Uncontrolled inflammation has been hypothesized to be a driver of poor outcomes after SAH [5]. The mechanisms triggering inflammatory cascades after SAH have been attributed to the breakdown of cells from the extravasated blood in the subarachnoid space [6]. Platelet activation has also been associated with worsening injury severity by complementing the spread of inflammation and perpetuating the cycle of injury expansion [7]. However, previous studies investigating pathophysiology after SAH have been limited for several reasons. First, many studies lack an appropriate control group; the presence of a cerebral aneurysm and the cytokine response associated with its treatment (mostly through surgical intervention) are not taken into account. Second, only a few isolated molecules are investigated in most studies. For example, IL6 [8], TNFα [9] and other general markers of inflammation have been shown to be elevated in SAH subjects with poor outcomes. However, many other cytokines involved in inflammation and thrombosis can contribute to hypercoagulability [10], angiogenesis [11], breakdown of the blood-brain barrier and vasogenic edema [12] and, they have not been thoroughly studied. Third, previous studies typically use a reductionist approach, examining one or a few molecules at a time – failing to account for complex associations among the molecules. The pathophysiologic response is complex and we believe that

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systematic approaches based on informatics techniques are required to delineate the different mechanisms [13]. It has been previously shown that such approaches can be used to identify cytokine clusters after SAH [14].

In this study, we characterized the systemic response to aneurysm rupture by comparing several cytokines - including those involved in inflammation, thrombosis, growth promotion and signaling - between patients with SAH, unruptured aneurysms (UA) and traumatic brain injury (TBI). The expression levels of these cytokines were compared across injury severity at admission and clinical status at discharge. Additionally, we used network models and clustering approaches to identify intercorrelated cytokine clusters and elucidate the associations among the identified clusters. We hypothesized that systemic levels of several inflammatory and growth factors were elevated after SAH. Additionally, we hypothesized that there were systematic differences in the patterns of cytokine associations that were unique to SAH.

2. Subjects, materials, and methods

2.1. Study population and patient criteria

This was a retrospective cohort study of patients with SAH, UA and TBI admitted to the neuroscience intensive care unit at the Memorial Herman Hospital-Texas Medical Center from July 2013 to March 2015. Plasma samples were collected within 24 h after SAH after surgical intervention. Non-aneurysmal SAH patients with trauma, arteriovenous malformation and mycotic aneurysms were excluded from the study. Patients with any condition(s) that could affect baseline inflammation, including history of malignancy and current pregnancy, were excluded.

Demographic and clinical information including the Hunt Hess (HH) score, occurrence of delayed cerebral ischemia (DCI) and functional outcomes at discharge using the modified Rankin scale (mRS) were collected prospectively. Subjects were dichotomized into good (HH ≤ 3) and poor (HH ≥ 4) clinical severity groups (see supplement for definitions of HH, DCI and mRS). Good clinical outcomes were defined as a mRS ≤ 3 and poor clinical outcomes were defined as a mRS ≥ 4.

2.2. Controls

We chose patients with UA and TBI as controls. UA subjects undergoing elective treatment for their aneurysm (incidental detection) were chosen because they had the same characteristics as the SAH subjects; presence of aneurysm and surgical interventions. Plasma samples were drawn from UA subjects during or after the elective procedure. Additionally, to contrast the response in SAH to other acute neurological injury, plasma samples from a secondary cohort of 77 TBI patients were included in the study.

2.3. Standard protocol approvals, registrations, and patient consents

The study was conducted with the approval of the institutional IRB ((IRB Number HSC-MS-12-0637). Written informed consent was obtained from the patient or surrogate.

2.4. Cytokine analysis

The cytokines analyzed in this study and the multiplex assay experimental protocol are listed as supplemental material (see supplemental).

2.5. Statistical analysis

Descriptive statistics were calculated for demographic variables and cytokine levels in SAH and control groups (Table S1 and Table 1) and between good and poor HH patients in the SAH group (Table 2). To describe differences in demographic variables between SAH and control groups, and across HH grades, χ2-test, Fisher's exact test, student's *t*-test, and the Mann-Whitney *U* test were used where appropriate. The Mann-Whitney *U* test was used to test for differences in cytokine levels across different groups. Statistical analyses were performed using open-source software packages in R (v3.1.3). Cytoscape (v3.2.1, Institute for Systems Biology, Seattle, Washington) was used for network visualization [15].

Table 1
Cytokine differences between UA and SAH.

Cytokines	Total	UA	SAH	*p-value*
	N = 83	N = 23	N = 60	
Cluster-A				
PDGF-AA	63(14–137)	25(9.2–60)	106(20–249)	< **0.01**
CCL2	251(175–363)	262(215–355)	225(154–364)	0.17
IP10	337(202–637)	509(336–731)	297(187–553)	**0.02***
sCD40L	52(36–93)	43(30–53)	65(40–106)	< **0.01***
CXCL1	174(108–382)	81(46–179)	230(143–411)	< **0.01***
CCL5	39(6.7–797)	4.9(1.3–8.01)	400(25–1117)	< **0.01***
PDGF-AB/BB	36(2.9–317)	2.7(1.9–4.02)	210(11.5–594)	< **0.01***
Cluster-B				
TNFa	5.1(4–7.8)	4.4(3.6–7.1)	6(4.3–8.2)	0.3
IL1A	2.5(0.32–63)	0.32(0.32–74)	3(0.3–63)	0.4
MIP1A	4.9(1–13.7)	6.8(2.8–12)	4.7(1–14)	0.2
VEGFA	87(28–257)	36(6.2–193)	111(36–278)	0.08
IL17A	3.58(0.9–8.8)	1.6(0.04–5.12)	4.2(1.5–9.7)	< **0.05***
IFNG	16.7(6–42)	15(3.3–24)	19(7.2–53)	0.18
CCL7	3.2(.9–23)	0.9(0.9–32)	10(0.9–23)	0.13
IL15	0.9(0.06–4.1)	0.06(0.06–0.41)	1.3(0.3–4.5)	< **0.01***
Cluster-C				
IL8	10(5–21)	4.1(1.6–9.9)	14.5(6.3–22.5)	< **0.01***
IL1R1	17(2.8–46)	0.6(0.03–16)	21(8.6–69)	< **0.01***
IL10	3.4(0.12–14)	0.12(0.12–6.9)	4.7(.6–14)	0.06
CSF3	38(17–74)	12.7(1.1–31)	42(24–91)	< **0.01***
CCL11	60(38–90)	74(44–99)	57(34–83)	0.125
IL6	7.4(1.9–18.4)	1.85(.14–6.6)	11.1(5.4–24.4)	< **0.01***
Cluster-D				
CX3CL1	44(2.7–149)	2(2–58)	57(28–168)	< **0.05***
TGFA	0.68(0.05–1.6)	0.05(0.05–0.05)	0.86(0.17–2.22)	< **0.01***
IL12p70	3.5(0.4–18)	0.8(0.15–6.2)	4.6(1–22)	**0.01***
MIP1B	20.4(10.2–37)	15(7.7–31)	21(11.5–39.6)	0.2
IL9	0.17(0.01–0.71)	0.01(0.01–0.33)	0.3(0.01–0.8)	0.2
EGF	8.1(0.7–23)	0.7(0.7–27)	9.7(1.4–23)	0.12
IFNA2	10.3*0.1–32)	0.1(0.1–9.2)	15.7(1.4–36.7)	< **0.05***
CSF2	3.88(0.11–13.7)	0.11(0.11–0.67)	9.2(1.8–14.2)	< **0.01***
FGF2	54(3–103)	3(3–59)	68(11–114)	< **0.01***
Others				
IL12p70	3.5(0.4–18)	0.8(0.15–6.2)	4.6(1–22)	**0.01***
FLT3L	0.76(0.76–3)	0.76(0.76–0.76)	1.3(0.76–5.14)	< **0.01***
IL12p40	0.5(0.5–11.7)	0.5(0.5–0.5)	0.5(0.5–14.3)	0.1
IL5	.2(0.02–1.09)	0.07(0.02–1.35)	0.25(0.04–0.9)	0.4
MIP1A	4.9(1–13.7)	6.8(2.8–12)	4.7(1–14)	0.2

Mann-Whitney *U* test was used to test for significance in differences across groups.

* Significant after adjusting for multiple comparisons using Benjamani and Hochberg method at a 10% false discovery rate.

2.6. Cluster analysis

To characterize the inflammatory response systematically, we used a network visualization framework and clustering techniques. A network consisted of nodes and edges. Each node represented a cytokine; the edges between the cytokines represented a correlation coefficient between them. The Box-Cox transformation was applied to normalize each distribution prior to computing the correlation coefficients. Non-normally distributed cytokines were excluded from the analysis based on the Kolmorgov-Smirnov and Shapiro Wilks tests. Cytokines that were undetectable in more than half the subjects were also excluded to