Stroke

EDITORIAL

Emerging Role of Neutrophil Extracellular Traps in Subarachnoid Hemorrhage

Jens Witsch, MD; Thilo Witsch, MD; Kimberly Martinod, PhD

In the days to weeks following spontaneous subarachnoid hemorrhage (SAH), up to 30% of patients develop brain infarcts, the imaging correlate of delayed cerebral ischemia (DCI).[1] DCI is of clinical relevance due to its high incidence, its demonstrated negative impact on outcome, and its delayed occurrence relative to the index SAH. The latter suggests a therapeutic window of opportunity.[1,2] However, DCI cannot be predicted due to a lack of reliable biomarkers, and prevention is centered on the use of the oral calcium antagonist nimodipine, which reduces the risk of DCI and poor outcomes. Other more effective treatments are unavailable because DCI's pathophysiology is not understood.[3] Anatomically, vasospasm of the larger brain vessels and, on a cellular level, neutrophil-mediated inflammation have been linked to secondary injury processes and DCI.[4,5] However, large-vessel vasospasm and DCI are correlated poorly,[6] and neutrophils have complex and essential roles in immunity, tissue repair, and resolution of inflammation. Thus, interventions targeting large-vessel vasospasm or broad blockade of neutrophil recruitment or function are not suitable treatment strategies.

See related article, p XXX

In this issue of *Stroke*, Nakagawa et al[7] present a study that follows both conceptual frameworks of DCI pathophysiology, vasospasm, and neutrophils, further downstream by investigating a potential association between microvasospasm and neutrophil extracellular traps (NETs) in a mouse model of SAH. NETs are DNA filaments decorated with cytotoxic and proinflammatory proteins, secreted by neutrophils into the extracellular space in response to pathogens or damage-associated molecular patterns. Maladaptive roles of NETs include thrombus formation, plaque destabilization, and endothelial dysfunction.[8–10]

Nakagawa et al developed a blood injection SAH mouse model combining it with in vivo 2-photon microscopy to visualize small brain vessels and the space and cells surrounding them. In their experiments conducted in 65 C57 black 6 adolescent mice, they confirmed that erythrocytes enter the perivascular space and gradually disappear over 2 to 5 days. Concomitantly, they found that neutrophils from the host's circulation infiltrated the perivascular space with timing, which coincided with the development of pearl-string–like microvasospasms of pial mouse vessels. The authors meticulously measured these microvasospasms using 2 methods, a visual and a semiautomated approach, with comparable results. Their study defines microvasospasm as a reduction of the vessel diameter of >20% and in a sensitivity analysis as a reduction of >40% (again, results are comparable). The main results of the study included that (1) antibody-mediated depletion of neutrophils significantly reduced vasospasm compared with isotype controls, (2) perivascular neutrophils released NETs (demonstrated by staining of key NET components, by intracisternally injected SYTOX Green staining, and by intracisternally injected fluorescein isothiocyanate–conjugated antineutrophil

Key Words: Editorials ■ cerebral infarction ■ extracellular traps ■ neutrophils ■ subarachnoid hemorrhage

Correspondence to: Jens Witsch, MD, Department of Neurology, Perelman School of Medicine, University of Pennsylvania, 9 Founders Bldg, 3400 Spruce St, Philadelphia, PA 19104. Email jens.witsch@pennmedicine.upenn.edu
For Sources of Funding and Disclosures, see page XXX.

Stroke is available at www.ahajournals.org/journal/str

elastase antibody and PE [phycoerythrin]-conjugated anti-H3 [histone H3] antibody, paired with subsequent in vivo perivascular visualization of the NETs components, as well as direct time-lapse visualization of neutrophils secreting NETs), and (3) NET degradation through intracisternal administration of exogenous DNase (deoxyribonuclease) slowly removed NETs from the perivascular space and significantly reduced microvasospasms in DNase-treated mice compared with controls.

Most notable is that Nakagawa's study suggests that NETs, a known driver of immunothrombosis, are located in the immediate surrounding of pial blood vessels after SAH in vivo; the presence of NETs is associated with microvasospasms; and NET depletion is associated with improvement of microvasospasms. The study results complement recent findings describing intravascular NETs in mice and peripheral blood NETs in humans post-SAH and implicate NETs in the pathophysiology of DCI.[11–13] Although more mechanistical insights are needed, Nakagawa et al's study supports the role of NETs as a potential treatment target to prevent secondary ischemia after SAH. The demonstration of perivascular NETs comes with the caveat that in vivo visualization of NETs is technically challenging and error-prone, as SYTOX Green staining is not NET-specific nor does it necessarily indicate extracellularly released DNA (compromised cell membranes also lead to SYTOX Green positivity in cells). However, the authors performed subsequent immunohistochemistry to identify specific neutrophil components and demonstrated a reduction in SYTOX Green signal after DNase administration, which indirectly suggests that the SYTOX Green–positive areas were, in fact, extracellular and of neutrophil origin.

From a clinical perspective, when trying to translate the findings in mice to human disease, the ubiquity of microvasospasm (and its association with NETs) complicates matters. Recent studies have also shown nearly ubiquitous microvasospasm in the pial arteries of mice after induced SAH.[14–16] One of these prior studies showed a reduction in vasospasm after intravenous administration of the iron scavenger deferoxamine, whereas Nakagawa et al now showed a reduction in microvasospasm after NET inhibition. While these results are not contradictory (iron from free heme may trigger neutrophil-mediated inflammation, which can result in NETs formation and secretion),[17] the ubiquity of microvasospasm in mice and the sporadic nature of DCI in humans beg the question of what other factors are needed to produce DCI. Do patients with SAH, like this mouse model, develop ubiquitous microvasospasm? If they do, why do only about one-third of patients develop macroscopically visible infarcts? Do the other two-thirds develop mini infarcts not visible on conventional clinical imaging? Or are microvasospasms contributing to ischemia-promoting machinery that produces infarcts only after an ischemic threshold has been surpassed? Is it the interplay between microvasospasm, the associated reduction in blood flow, and intravascular thrombosis (possibly driven by intravascular NETosis) that leads to DCI? Future studies have many questions to answer, for which Nakagawa et al's work has opened the door.

ARTICLE INFORMATION

Affiliations

Department of Neurology, Hospital of the University of Pennsylvania, Philadelphia (J.W.). Department of Cardiology and Angiology, University Heart Center Freiburg - Bad Krozingen, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Germany (T.W.). Division of Experimental Cardiology, Department of Cardiovascular Sciences, KU Leuven, Belgium (K.M.). Aab Cardiovascular Research Institute, University of Rochester Medical Center, NY (K.M.).

Sources of Funding

Dr Witsch received career development grant funding from the American Heart Association (https://doi.org/10.58275/AHA.23CDA1053561.pc.gr.168057) and the National Institutes of Health/National Institute of Neurological Disorders and Stroke (grant 1K23NS131588-01A1).

Disclosures

Dr Martinod is an inventor of granted patent US9642822B2 covering the targeting of NETs in thrombosis and received consulting fees from and holds stock options in Peel Therapeutics, Inc. The other authors report no conflicts.

REFERENCES

1. Claassen J, Park S. Spontaneous subarachnoid haemorrhage. *Lancet*. 2022;400:846–862. doi: 10.1016/S0140-6736(22)00938-2
2. Witsch J, Frey HP, Patel S, Park S, Lahiri S, Schmidt JM, Agarwal S, Falo MC, Velazquez A, Jaja B, et al. Prognostication of long-term outcomes after subarachnoid hemorrhage: the FRESH score. *Ann Neurol*. 2016;80:46–58. doi: 10.1002/ana.24675
3. Hoh BL, Ko NU, Amin-Hanjani S, Chou SY, Cruz-Flores S, Dangayach NS, Derdeyn CP, Du R, Hanggi D, Hetts SW, et al. 2023 guideline for the management of patients with aneurysmal subarachnoid hemorrhage: a guideline from the American Heart Association/American Stroke Association. *Stroke*. 2023;54:e314–e370. doi: 10.1161/STR.0000000000000436
4. Friedrich V, Flores R, Muller A, Bi W, Peerschke EI, Sehba FA. Reduction of neutrophil activity decreases early microvascular injury after subarachnoid haemorrhage. *J Neuroinflammation*. 2011;8:103. doi: 10.1186/1742-2094-8-103
5. Al-Mufti F, Amuluru K, Damodara N, Dodson V, Roh D, Agarwal S, Meyers PM, Connolly ES Jr, Schmidt MJ, Claassen J, et al. Admission neutrophil-lymphocyte ratio predicts delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage. *J Neurointerv Surg*. 2019;11:1135–1140. doi: 10.1136/neurintsurg-2019-014759
6. Suwatcharangkoon S, De Marchis GM, Witsch J, Meyers E, Velazquez A, Falo C, Schmidt JM, Agarwal S, Connolly ES, Claassen J, et al. Medical treatment failure for symptomatic vasospasm after subarachnoid hemorrhage threatens long-term outcome. *Stroke*. 2019;50:1696–1702. doi: 10.1161/strokeaha.118.022536
7. Nakagawa R, Itokazu T, Shibuya N, Kishima H, Ymashita T. Perivascular neutrophil extracellular traps exacerbate microvasospasm after experimental subarachnoid hemorrhage. *Stroke*. 2024;55:XX–XX. doi: 10.1161/STROKEAHA.124.047574
8. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. *Blood*. 2014;123:2768–2776. doi: 10.1182/blood-2013-10-463646
9. Cao J, Roth S, Zhang S, Kopczak A, Mami S, Asare Y, Georgakis MK, Messerer D, Horn A, Shemer R, et al; DEMDAS Study Group. DNA-sensing inflammasomes cause recurrent atherosclerotic stroke. *Nature*. 2024;633:433–441. doi: 10.1038/s41586-024-07803-4
10. Sorvillo N, Cherpokova D, Martinod K, Wagner DD. Extracellular DNA NET-Works with dire consequences for health. *Circ Res*. 2019;125:470–488. doi: 10.1161/circresaha.119.314581
11. Witsch J, Spalart V, Martinod K, Schneider H, Oertel J, Geisel J, Hendrix P, Hemmer S. Neutrophil extracellular traps and delayed cerebral ischemia in

aneurysmal subarachnoid hemorrhage. *Crit Care Explor*. 2022;4:e0692. doi: 10.1097/CCE.0000000000000692
12. Hendrix P, Witsch J, Spalart V, Schneider H, Oertel J, Geisel J, Martinod K, Hemmer S. Neutrophil extracellular trap biomarkers in aneurysmal subarachnoid hemorrhage: early decline of DNase 1 activity associated with delayed cerebral ischemia. *Front Neurol*. 2024;15:1354224. doi: 10.3389/fneur.2024.1354224
13. Zeineddine HA, Hong SH, Peesh P, Dienel A, Torres K, Thankamani Pandit P, Matsumura K, Huang S, Li W, Chauhan A, et al. Neutrophils and neutrophil extracellular traps cause vascular occlusion and delayed cerebral ischemia after subarachnoid hemorrhage in mice. *Arterioscler Thromb Vasc Biol*. 2024;44:635–652. doi: 10.1161/ATVBAHA.123.320224
14. Lin X, Khalin I, Harapan BN, Terpolilli NA, Schwarting J, Plesnila N. Perivascular macrophages mediate microvasospasms after experimental subarachnoid hemorrhage. *Stroke*. 2023;54:2126–2134. doi: 10.1161/strokeaha.122.042290
15. Liu H, Schwarting J, Terpolilli NA, Nehrkorn K, Plesnila N. Scavenging free iron reduces arteriolar microvasospasms after experimental subarachnoid hemorrhage. *Stroke*. 2021;52:4033–4042. doi: 10.1161/STROKEAHA.120.033472
16. Schwarting J, Nehrkorn K, Harapan BN, Balbi M, Terpolilli NA, Plesnila N. Pericytes are not associated with reduced capillary perfusion after experimental subarachnoid hemorrhage. *Stroke*. 2023;54:2172–2177. doi: 10.1161/STROKEAHA.123.042936
17. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood*. 2014;123:3818–3827. doi: 10.1182/blood-2013-10-529982

EDITORIAL