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DAMPs converging on Toll-like receptor 4 in hemorrhagic stroke, a Mini-Review.

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Abstract

Hemorrhagic stroke, resulting from a brain aneurysm or weakened blood vessel rupture, accounts for only 15-20% of all strokes, yet is responsible for nearly half of all stroke-related mortality. Toll-like receptor 4 (TLR4), which senses highly conserved pathogen/danger associated molecular patterns (PAMPs/DAMPs) present on pathogens and in host tissue following injury, contributes to poor outcome in rodent models of hemorrhagic stroke. There is growing evidence that neuronal injury, physical disability and mortality is a result of perihematomal neuroinflammation mediated by activation of TLR4. Several molecules present in high quantities following hemorrhage have been purported to activate TLR4. However, the possibility of lipopolysaccharide (LPS) and lipopeptide contaminants have confounded our understanding of these molecules as genuine TLR4 ligands or as inflammatory sensitizing agents. In this review, we describe the temporal convergence of DAMPs on TLR4 in hemorrhagic stroke and highlight the available evidence for each as a mediator of neuroinflammation.

Keywords: subarachnoid hemorrhage; intracerebral hemorrhage, toll-like receptor 4, lipopolysaccharide

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Introduction

Hemorrhagic stroke results from rupture of a brain aneurysm or weakened blood vessel; Introducing normally sequestered intravascular contents into the subarachnoid space or brain parenchyma. For reasons incompletely understood, this type of insult is particularly devastating to the brain. Hemorrhagic stroke accounts for only one in every five strokes, yet it is responsible for nearly half of all stroke-related mortality. Moreover, surviving patients are nearly twice as likely to become cognitively disabled compared to patients with ischemic stroke [1]. Progressive cell death and/or necrosis from ischemia initiates inflammatory cascades – but the presence of hemoglobin and other factors in hemorrhagic stroke may underlie the predominating neuroinflammatory response to hemorrhage and account for the major differences in morbidity and mortality. A growing number of studies have described the presence of an intense inflammatory response surrounding the hemorrhage, and have implicated this immune response in the pathogenesis of secondary injury after hemorrhagic stroke. In the hours to days following hemorrhage, the innate inflammatory response to extravasated blood leads to microglial activation, cytokine production, neutrophil recruitment and neuronal loss, all of which contribute to poor outcome and long-term cognitive dysfunction [1]. Understanding the events that initiate or propagate the inflammatory response may lead to therapies that improve clinical outcomes.

Notably, persistent activation of Toll-like receptor 4 (TLR4) in the brain is one of the few molecular mechanisms established as a cause of long-lasting cognitive dysfunction [2]. TLR4 recognizes pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) and plays a central role in innate immunity. Activation of TLR4 expressed on microglia and infiltrating leukocytes are likely the most important sources of chronic neuroinflammation in hemorrhagic stroke; the contribution of neuronal, astrocytic and endothelial TLR4 is not well-established [1].

While TLR4 is critical for host responses to pathogens, it also contributes to the development of sterile inflammation by binding directly to endogenous proteins that act as "damage signals" following injury [3]. An additional class of endogenous proteins released following injury are better described as PAMP-sensitizing or PAMP-binding molecules (PSMs, PBMs), which act on TLR4 indirectly. PSM/PBMs have the capacity to interact cooperatively with PAMP/DAMPs, strengthening the effect of genuine TLR4 ligands [3]. Notably, even the classic TLR4 ligand, bacterial lipopolysaccharide (LPS), not only requires

additional binding factors in order to fully interact with the TLR4 complex [for example, LPS-binding protein (LBP)], but can also be potentiated by other molecules (for example, β-amyloid and heat-shock proteins) [3-5]. Thus, in order to better study the molecular events involved in hemorrhage-induced neuroinflammation, a more complete analysis of DAMPs, PSMs and PBMs must be made.

The role of TLR4 in hemorrhage-induced neuroinflammation is now well-established [1]. There are at least five molecules present in the brain after hemorrhagic stroke that have been postulated to be endogenous TLR4 ligands: (i) hemoglobin (and metabolites); (ii) fibrinogen; (iii) MRP8/14; (iv) Heat-shock proteins (HSPs) and (v) HMGB1. This review will focus on the temporal convergence of these molecules on TLR4 following hemorrhagic stroke, and highlight the evidence for each as a mediator of neuroinflammation.

Limitations of widely used anticontamination practices

The evaluation of new TLR4 ligands would not be complete without a discussion of common methodology used to validate experimental results. Several methods are frequently employed to establish that the effects of a putative TLR4 ligand are not due to contaminating LPS or lipopeptides, each with notable and often overlooked limitations [3]. The *Limulus* Amoebocyte Lysate (LAL) assay to detect contaminating LPS is most widely used, but is easily confounded by the presence of molecules that bind LPS, including PBMs. Thus, it is possible that the LPS content of many proposed ligands could have been greatly underestimated if these ligands have any capacity to bind LPS. Moreover, the LAL assay is not able to detect lipopeptide contaminants, which may also have the capacity to bind to TLR4. Polymyxin B is also used frequently as a well-characterized molecule that sequesters LPS of enterobacterial origins. Notably, an often overlooked concern of polymyxin B is that it does not inhibit the signaling by LPSs of several non-enterobacterial organisms. Moreover, there is evidence that PBMs can effectively shield LPS from the inhibitory effects of polymyxin B, a further confounder.

Other popular anti-contamination methods are heat denaturation and protease treatment, to which LPS is expected to have some resistance [3]. Contrary to popular belief, however, the biological activity of LPS is in fact reduced significantly by heating. Moreover, PBMs or other factors that serve to enhance sensitivity to low levels of LPS contamination may lose their capacity to do so following heat or protease treatment. Thus, some caution is required when interpreting results of experiments employing these common control practices. These considerations suggest that if any proposed TLR4 ligand has any PSM of PBM capacity, then it may be difficult to discount potential contamination of such molecules by the use of LAL assays, PMB, protease, or heat treatment alone [3].

Hemoglobin and metabolites

The leading event of hemorrhagic stroke is the extravasation of blood beyond the blood-brain barrier. Erythrocyte hemolysate, some components of which mimic adverse consequences of hemorrhagic stroke, contains three purported endogenous TLR4 ligands: methemoglobin (metHgb[Fe^{3+}]), heme and hemin [3-6]. A fourth ligand, oxyHgb[Fe^{2+}], has also been experimentally demonstrated to activate TLR4, but it is likely that spontaneous oxidation under normal culture conditions would have converted Hgb to metHgb. Poor water solubility of heme and hemin, and potential contamination of metHgb by LPS confounded our previous understanding of the potential role of hemolysate products as endogenous TLR4 ligands [2,3].

We recently used a 5-step process to obtain ultra-pure, LPS-free metHgb from hemolysate, confirmed using a 12 Tesla Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer. Using this preparation, we established that metHgb is an endogenous TLR4 ligand at physiological concentrations.[2] In cultures of TLR4-expressing microglia, we found that metHgb induced nuclear translocation of NF-κB, a hallmark of TLR4 activation, as well as a time- and dose-dependent secretion of TNFα. Completive, non-competitive and genetic inhibition of TLR4 all abrogated the response of microglia to metHgb. Of note, we compared microglial TNFα secretion in cells that were treated with LPS-free hemolysate versus LPS-free metHgb, and found that >98% of the TLR4-activating efficacy of hemolysate could be accounted for by metHgb. Purified LPS-free metHgb was also infused into the subarachnoid space of the entorhinal cortex of rats [2]. Immunolabeling of brain sections for ionized calcium binding adaptor molecule 1 (Iba1) and TNFα showed robust microglial activation in the adjacent entorhinal cortex as well as remotely in the hippocampus. Immunolabeling also showed that microglial activation was accompanied by microglial upregulation of TLR4.

Similar, rigorous experimental approaches should be performed in order to establish the precise activity of heme and/or hemin on TLR4. Many putative TLR4 ligands demonstrate no intrinsic TLR-stimulating potential when highly purified reagents are examined, and could best be described as cooperative PSMs/PBMs [3]. Our work confirmed that metHgb was sufficient to induce neuroinflammation in a model of subarachnoid hemorrhage and should be included among the endogenous TLR4 ligands.

Fibrinogen

Another component of extravasated blood, fibrinogen, may also contribute to hemorrhage-induced neuroinflammation. Fibrinogen is converted to fibrin following activation of

the coagulation cascade, which is then deposited at the site of extravasation. Fibrin(ogen) is well-known to participate in leukocyte trafficking via binding to specific integrins on the cell surface of inflammatory cells.

Extravascular fibrinogen may also function by transmitting activating signals to inflammatory cells via TLR4 ligation. Smiley et al. studied the effect of fibrinogen on TLR4-dependent chemokine release [7]. They observed that RAW264.7 macrophages significantly upregulated mRNA encoding for several chemokines when treated with fibrinogen. Additionally, fibrinogen dose-dependently induced the release of MCP-1 in both RAW264.7 and primary mouse macrophages. For their experiments, commercially available fibrinogen was purchased from three available vendors; <1 U/mL contaminating endotoxin was measured via LAL assay for each. To establish that fibrinogen-induced chemokine secretion could not be explained by LPS contamination, the authors supplemented cultures with polymyxin B and also evaluated heat-denatured fibrinogen. Polymyxin B had no effect on MCP-1 induction by fibrinogen compared to heat-denaturation, which significantly reduced induction. They also examined the effects of fibrinogen in macrophages derived from TLR4-deficient mice, and found that fibrinogen failed to induce MCP-1 release. Although the authors could not rule out contamination of heat-sensitive, polymyxin B resistant LPS, taken together, the authors concluded that the effect of fibrinogen on chemokine induction was LPS independent and TLR4 dependent.

Fibrinogen has also been demonstrated to act as a PSM and PBM. Nakatomi et al. studied the mode of action of plasma-mediated immobilization of LPS to plastic surfaces [8]. They found that fibrinogen effectively immobilized LPS in an LPS binding protein-independent fashion. Additionally, they demonstrated that fibrinogen could prime neutrophils toward a greater responsiveness to LPS. Thus, it is possible that fibrinogen acts both as an endogenous TLR4 agonist and as a cooperative sensitizing molecule. Further studies using highly purified fibrinogen will determine the specific role of fibrinogen in TLR4-mediated neuroinflammation in hemorrhagic stroke.

Myeloid-related protein (MRP) 8/14

Neutrophil recruitment to the site of hemorrhage is another early and prominent feature of hemorrhagic stroke. MRP-8 (S100A8) and MRP-14 (S100A9) are the most abundant cytoplasmic proteins of neutrophils and complexes of MRP-8/14, the most physiologically relevant form of these proteins, are specifically released during activation. MRP-8/14 released from activated neutrophils is capable of inducing a robust inflammatory response [5]. Although MRP-8/14 has not been explicitly evaluated in hemorrhagic stroke, the role of neutrophils in propagating hemorrhage-induced neuroinflammation is certain, as several studies have demonstrated anti-inflammatory and neuroprotective effects of neutrophil depletion (see refs 17 and 18 in 1).

Vogl and colleagues explored the extracellular function of MRP-8/14 in order to characterize the mechanism of an MRP-8/14-mediated inflammatory response. They found that MRP-8/14 is an endogenous ligand of TLR4, and, more precisely, that MRP-8 is the active component. They observed that MRP-8 induced TLR4 signal transduction via ERK/MEK, p38 MAP kinase and protein kinase C, could induce MyD88 translocation to the cell membrane, and could induce TNFα secretion, in similar fashion to LPS.[5] Moreover, MRP-8 had no stimulatory effect on macrophages derived from mice expressing non-functional TLR4. The authors took several complimentary steps in order to strictly control for contamination of LPS in their protein. Using the LAL assay, they could not detect contaminating LPS (kit sensitivity < 1 pg/μg protein). Polymyxin B (25 μg/mL) had no effect on the stimulatory effect of MRP-8, while it abolished the effect of LPS up to 10 ng/mL (1,000-fold higher than the maximal trace LPS contamination). Heat-inactivation abolished the effect of MRP-8, whereas LPS was not inactivated at the temperature used to denature MRP8. Perhaps the most convincing evidence that MRP-8/14 is an endogenous ligand of TLR4 came from the results of their plasmon resonance experiments, which demonstrated a direct and strong interaction between the TLR4-MD2 complex.

Of note, MRP-8/14 has been observed to exhibit lipid/lipoprotein binding capacity (reviewed in [3]), which could serve as TLR4 ligands on their own. Contamination of this type would not be detectable via LAL assay. Although it has not been directly tested, the ability of MRP-8/14 to interact with lipids/lipoprotein suggests it could also for complexes with other DAMPs. Evidence that MRP-8/14 may serve as a PBM or PSM also comes from Vogl et al., where they demonstrated amplification of LPS-induced signaling with concurrent MRP-8/14 treatment [5]. Given these apparent dual roles of MRP-8/14, and the role of neutrophils as mediators of acute neuroinflammation, the study of MRP-8/14 in hemorrhagic stroke should garner further attention.

Heat Shock Proteins (HSP)

HSP, which are normally sequestered in the cytoplasm, are a family of proteins that are produced by cells following exposure to stressful conditions or during injury. As tissue injury progresses in the hours to days following hemorrhagic stroke, necrotic cells release sequestered intracellular HSP into the extracellular milieu. HSP, in particular HSP60 and HSP70, have been purported to be endogenous TLR4 ligands (see refs 18-21 in [3]), with relevance in hemorrhagic stroke (reviewed in [1]). However, it is now widely considered that the original characterization of HSP as TLR4 ligands was due not to intrinsic activity, but rather due to contaminating LPS in

the recombinant *E. coli*-expressed HSP preparations [4,9].

Nonetheless, continued interest in HSPs as modulators of inflammatory signaling prompted additional inquiry. Several studies, including that of Osterloh et al., demonstrated that HSP60 functions both as a synergistic PBM and a potentiating PSM [4]. They recapitulated earlier findings, confirming that HSP60 specifically binds to LPS and activates macrophages in a synergistic manner. Perhaps more relevant in the context of sterile hemorrhage-induced neuroinflammation, they also found that in macrophages, HSP60 co-localized to LPS binding sites and the LPS co-receptor CD14, in a distinct pattern that might represent lipid raft-like membrane regions. This is notable because LPS binding to CD14 is known to initiate co-assembly of the larger TLR4 complex, a required prerequisite for signal transduction [1,3]. Thus, HSP60 release from necrotic cells might enhance or prolong hemorrhage-induced neuroinflammation by promoting clustering of lipid-raft domains and full assembly of the TLR4 signaling complex.

High-mobility group protein B1 (HMGB1)

HMGB1, a DNA-binding protein found both in the nucleus and cytoplasm under normal conditions, is actively secreted by stimulated immune cells and passively released from injured cells, including neurons following hemorrhagic stroke.[3,10] Like HSP, extracellular HMGB1 is believed to play a role in the progression of TLR4-dependent hemorrhage-induced neuroinflammation (see refs 91 and 92 in [1]). HMGB1 was initially characterized as an endogenous ligand of TLR4. However, more recent studies using low endotoxin rHMGB1 derived from mammalian expression systems revealed no capacity of this molecule to stimulate TLR4 (reviewed in [3]). Nonetheless, there is mounting evidence that HMGB1 participates in TLR4 signaling as a potent PSM and PBM, specifically acting in synergy with hemoglobin [10]. Thus, HMBG1 remains an intriguing therapeutic target in hemorrhagic stroke [1,10].

Lin and colleagues studied the interactions of hemoglobin and HMGB1 on the activation of macrophages. Using low-endotoxin preparations (<0.01 EU/mg protein), they found that these molecules acted synergistically to enhance cytokine production at concentrations that likely far exceeded those in the hemorrhagic milieu.[10] While this synergy was multifactorial, they found a clear contribution of TLR4, as the synergistic response was significantly blunted in macrophages derived from *Tlr4 –/–* mice. Interestingly, they also found “3-way synergy” with additional PAMPs, which may more closely mimic the conditions found in nature following injury. The exact mechanism of the synergy between HMGB1 and hemoglobin is unclear. However, in the case of HMGB1-LPS synergy, HMGB1 appears to function as a catalyst for CD14 binding, and thus TLR4 complex assembly [3]. Thus, in the setting of hemorrhagic stroke, the most likely role of HMGB1 is as a PSM or chaperone PBM.

Conclusion

Molecules associated with danger, damage and injury are abundant in hemorrhagic stroke, and prolonged inflammation is a critical determinant of the long-term cognitive dysfunction observed in survivors of this type of insult. Notably, persistent activation of TLR4 in the brain is one of the few molecular mechanisms established as a cause of long-lasting cognitive dysfunction. The earliest and most abundant DAMPs come directly from extravasated blood. Shortly after, inflammatory cells recruited to the site of hemorrhage become activated, releasing soluble factors that also converge on TLR4. Concurrently, irreversible damage the CNS takes places, flooding the extracellular milieu with the components of injured and necrotic cells, further propagating TLR4-mediated neuroinflammation. Studying the interaction and synergy between all of these temporally related signals is critical if we are to fully understanding the events that initiate or propagate the inflammatory response to hemorrhage. Only then may our efforts lead to therapies that improve clinical outcomes for this devastating injury.

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