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Microglial Polarization and Inflammatory Mediators After Intracerebral Hemorrhage

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Abstract Intracerebral hemorrhage (ICH) is a subtype of stroke with high mortality and morbidity. When a diseased artery within the brain bursts, expansion and absorption of the resulting hematoma trigger a series of reactions that cause primary and secondary brain injury. Microglia are extremely important for removing the hematoma and clearing debris, but they are also a source of ongoing inflammation. This article discusses the role of microglial activation/polarization and related inflammatory mediators, such as Toll-like receptor 4, matrix metalloproteinases, high-mobility group protein box-1, nuclear factor erythroid 2-related factor 2, heme oxygenase, and iron, in secondary injury after ICH and highlights the potential targets for ICH treatment.

Keywords Intracerebral hemorrhage · Microglia · Inflammatory mediators

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Introduction

Intracerebral hemorrhage (ICH) accounts for 15–20 % of all strokes and is associated with high morbidity and mortality [1]. During ICH, rapid accumulation of blood within the surrounding brain leads to primary injury [2]. This primary damage occurs within minutes to hours from the onset of the event, causes disruption of normal anatomy, and leads to high pressure in the local brain tissue [2]. Secondary damage is, for the most part, due to the presence and clearance of hematoma [1–3]. Inflammatory cells, including blood-derived leukocytes, microglia/macrophages, astrocytes, and mast cells, are vital for clearing the hematoma but can also augment the brain damage caused by ICH [3–5]. Microglia are considered to be the earliest inflammatory cells to react to ICH. Increasing evidence suggests that microglia are the major cell type responsible for secondary damage after ICH owing to their release of cytokines, chemokines, prostaglandins, proteases, ferrous iron, and other immunoactive molecules [6–9]. In this review, we will discuss the role of microglial activation/polarization and summarize recent progress in the study of inflammatory mediators, such as Toll-like receptor 4, matrix metalloproteinases, high-mobility group protein box-1, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase (HO), and iron, which are released by microglia during the secondary injury phase after ICH.

Microglia

Microglial Activation After ICH

Microglia are the critical immune cells in the central nervous system (CNS) and are often referred to as the brain's macrophage. They are involved in brain development, maintenance of the neural environment, and response to injury and repair

[10–12]. Microglia can be activated by various types of brain damage and undergo phenotype and functional transformations to maintain tissue homeostasis. Specifically, activated microglia become enlarged, with rod-like, spherical, or amoeboid shapes, and phagocytic [13]. Because both microglia and macrophages originate from primitive myeloid progenitors, they express the same markers (CD11b, F4/80, Iba-1) [14]. For this reason, it is difficult to distinguish activated microglia from activated macrophages by immunohistochemistry. Therefore, most researchers customarily identify activated microglia as microglia/macrophage. However, studies with flow cytometry reported that microglia and macrophages can be separated based on the expression of $CD45^{low}CD11b^{+}$ in microglia [15–17].

Microglia are the first immune cells to react to brain injury and can be activated by blood components, including red cells, heme, leukocytes, and plasma proteins. The activation of microglia is thought to have a dual role in ICH. The main role of activated microglia is to phagocytose the hematoma and cell debris, thereby maintaining tissue homeostasis, and to promote neurologic functional recovery. However, in this process, the activated microglia can also produce a variety of deleterious cytokines, such as proinflammatory factors, chemokines, reactive oxygen species (ROS), proteases, nitric oxide (NO) synthase, and prostaglandins, which increase brain damage after ICH [6, 18–23].

Knowledge of microglial activation during ICH comes mainly from preclinical animal models. Though many different animal models of ICH have been established [24], the most frequently used are the autologous blood-induced and collagenase-induced models in rodents [25]. Both models have limitations in their ability to reflect the exact clinical features of ICH, but they are currently the most suitable tools to study the mechanism of ICH [26]. The advantage of the autologous blood model is that only blood is injected into the striatum. This model is appropriate for studying mechanisms of neuronal damage induced by blood and blood catabolism. We modified this method by injecting the blood in two portions to prevent the blood from flowing back along the needle track [27]. However, this model does not mimic brain damage caused by hematoma growth, which occurs clinically in approximately 73 % of patients [28]. The second commonly used model, the collagenase injection model, is better able to mimic acute cerebrovascular injury and hematoma growth. However, the bleeding results from the breakdown of cerebral blood vessels rather than the rupture of a small, deep-penetrating artery, as is seen in patients [26, 29, 30]. Moreover, whether the collagenase can directly induce inflammation is still disputed [26, 29, 31]. Because the two models reflect different pathomechanisms of ICH, we use both to study the mechanisms and roles of genes and proteins and to evaluate the effectiveness of drugs [9, 21, 32].

Activated microglia can be detected within 1 h after collagenase-induced ICH, even earlier than the appearance of neutrophils, which are seen within 4–5 h after collagenase injection [33]. As in the collagenase model, activated microglia can be found in the perihematoma within 1–4 h in the autologous blood model [34]. In both models, the peak microglial activation is within 3–7 days [29, 35]. The number of microglia decreases after 7 days and returns to normal at 21 days [29], although some studies show that microglial activation will persist for 4 weeks [26, 36]. The time course of microglial activation may determine their different roles in the process of ICH. In the early stage of ICH, activated microglia have been shown to produce proinflammatory factors and the numbers of microglia in the ipsilateral hemisphere correlate with neurologic functional damage [37]. Additionally, inhibiting microglial activation can decrease brain injury and edema [38]. Compounds that regulate microglial activation, such as sinomenine [20], minocycline [39], microglia/macrophage inhibitory factors (tuftsin fragment 1–3) [29, 40], misoprostol [21], Chinese medicine (curcumin [41], sesamin [42]), and iron chelators deferoxamine [43, 44] and 2,2′-dipyridyl [45], reduce ICH-induced brain injury and improve neurologic function in rodents. In addition, cell therapies, including mesenchymal stem cells [46] and regulatory T cells [47], attenuate ICH-induced brain injury, which correlates with inhibition of microglial activation. However, the underlying signaling pathways involved in microglia-regulated inflammatory responses after ICH remain elusive.

Microglial Polarization

With appropriate stimulation, both microglia and macrophages can be activated to two polarization states: the M1, classically activated phenotype and the M2, alternatively activated phenotype. Stimulation with lipopolysaccharide or interferon gamma [48] causes microglia to be activated to the M1 phenotype and to produce proinflammatory mediators (interleukin (IL)-1β, IL-6, IL-12, IL-23, tumor necrosis factor alpha (TNF-α)), chemokines, redox molecules (NADPH oxidase, phagocyte oxidase, inducible NO synthase), costimulatory proteins (CD40), and major histocompatibility complex II (MHC-II) [49–53]. Microglia can also be activated by IL4/IL3 to the M2 polarization state, which produces anti-inflammatory mediators (IL-10, transforming growth factor beta (TGFβ), and glucocorticoids) [54, 55]. M2 microglia are also interpreted to be nerve repair cells, as they secrete anti-inflammatory factors and upregulate neuroprotective factors in CNS disease [56]. Because M1 and M2 microglia secrete different factors, several markers have been used to distinguish the two phenotypes. MHC-II, CD16, CD32, CD80 (B7-1), CD86 (B7-2), and CD40 (TNFR) are commonly used as markers for M1 microglia [16, 57–64]. The alternatively activated M2 cells also present several specific

antigens, including Ym-1 (chitinase 3-like 3), CD206 (mannose receptor), CD68, and arginase-1 [16, 65].

Although the complexity of the microglial phenotype shift after injury requires additional research to decipher, it appears that that one phenotype can convert to the other. After permanent middle cerebral artery occlusion in mice, M2 markers CD206 and Ym-1 were found exclusively in the ischemic core at 24 h, but M1 markers CD16, CD32, and CD86 increased over time beginning on day 3 and outnumbered the M2 cells during the second week [66]. These results reveal a dynamic change of M2 to M1 phenotype over time in the ischemic brain. The early recruitment of M2 microglia/macrophages may be an endogenous response to clear toxic waste products and protect the ischemic brain. But, this change is transient. In time, the M1 microglia/macrophages become dominant in the injury area and exacerbate brain damage by promoting an inflammatory response. This change from an M2 to an M1 phenotype has also been reported in models of traumatic brain injury [67] and spinal cord injury [68]. Moreover, induction of microglia/macrophage M2 polarization is considered to be beneficial for the damaged brain. For example, IL-4 administration can promote M2 polarization and reduce ischemic lesion volume [69] and lipopolysaccharide preconditioning improves spinal cord injury by facilitating M2 activation [70]. Although promoting microglia/macrophage M2 polarization could be beneficial for brain recovery, the phenotypic change of microglia during ICH has not been confirmed. Owing to their phenotypic duality, it is thought that microglia may also have both proinflammatory and neuroprotective roles after ICH (Fig. 1).

Inflammatory Response Mediated by Microglia After ICH

Recently, Sansing et al. [71] showed that activated microglia express high levels of Toll-like receptor (TLR) 4, which leads to neuroinflammation after ICH. TLRs belong to a pattern-recognition receptor family and play an important role in innate immunity and inflammatory responses [72, 73]. To date, ten functional TLRs have been found in humans and 13 in mice. TLRs can recognize distinct pathogen-associated molecular patterns from viruses, bacteria, mycobacteria, fungi, and parasites. TLR4 in particular contributes to inflammatory injury in CNS pathologies such as cerebral ischemia and ICH [74–76]. Treatment with the TLR4 inhibitor ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl) sulfamoyl]-cyclohex-1-ene-1-carboxylate (TAK-242) or genetic deletion of TLR4 in mice decreases inflammation-mediated brain damage and improves neurologic function after ICH [71, 77–80]. Recent clinical studies also have shown that increased expression of TLR4 is associated with poorer functional outcome and greater residual volume in ICH patients [81]. Compared with wild-type (WT) mice, TLR4 knockout ($^{-/-}$) mice exhibited less water content and fewer neurologic deficits [71]. What is more, infiltrating inflammatory cells (macrophages, leukocytes, and monocytes) and inflammatory cytokines (TNF-α, IL-1β, and IL-6) were decreased around the perihematomal region in TLR4$^{-/-}$ mice [71, 77, 79].

TLRs bind various ligands through the leucine-rich repeats in their extracellular ectodomains and then recruit intracellular adaptor proteins, including myeloid differentiation factor 88 (MyD88), Toll-interleukin 1 receptor (TIR) domain-containing adaptor-inducing interferons (TRIFs), TIR domain-containing adaptor protein (TIRAP), and TRIF-related adaptor molecule (TRAM), by their intracellular TIR domains [78]. These adaptors then upregulate transcription factors such as NF-κB to promote microglial secretion of IL-6, TNF-α, and IL-1β [72, 78]. Studies have shown that MyD88 and TRIF are the main signaling pathways of TLR4-induced inflammatory response [78]. Both MyD88 and TRIF signals participate in the regulation of inflammatory factors after ICH. MyD88 or TRIF gene deficiency causes a decrease in the secretion of inflammatory factors and infiltration of microglia/macrophages after ICH [77]. The expression of MyD88 and TRIF is also decreased after ICH in TLR4$^{-/-}$ mice [77]. In short, the activation of microglia may mediate an inflammatory response in the early stage of ICH through TLR4 and its downstream signaling. A deeper understanding of the TLR4 signaling pathways should enable development of potential therapeutic targets for prevention and treatment of ICH.

Matrix metalloproteinases (MMPs) are a large family of ubiquitous zinc-dependent endopeptidases. Currently, 24 MMP genes and 23 MMP proteins have been identified in humans, with two identical genes on chromosome 1 that encode MMP-23 [82]. Under normal conditions, the MMP expression is low but it can be activated in response to various brain diseases [82, 83]. Studies of MMPs in ICH have focused mainly on MMP-2 and MMP-9, which increase in mouse brain within 2–3 days after ICH [84, 85]. The elevations in expression or activation of MMP-2 and MMP-9 correlate highly with brain edema, inflammation, and blood–brain barrier disruption, and these effects can be reduced by MMP inhibition [21, 84, 85]. In acute stroke, activation of MMP-2 initially disrupts the extracellular matrix proteins in the basal lamina and then attacks the tight junction proteins. If the hypoxic stress does not continue, or MMP is not further activated, the basal lamina integrity can be restored [83]. If damage continues, MMP-9 can be activated by neuroinflammatory cytokines (TNF-α and IL-1β) and free radicals. MMP-9 then degrades the basal lamina and tight junctions of endothelia cells, causing BBB disruption and vasogenic edema [83, 84]. Although the exact relation between MMP-9 and microglia is still unclear, microglia are one of the major sources of MMP-9. The cytokines (TNF-α and IL-1β) and free radicals secreted by

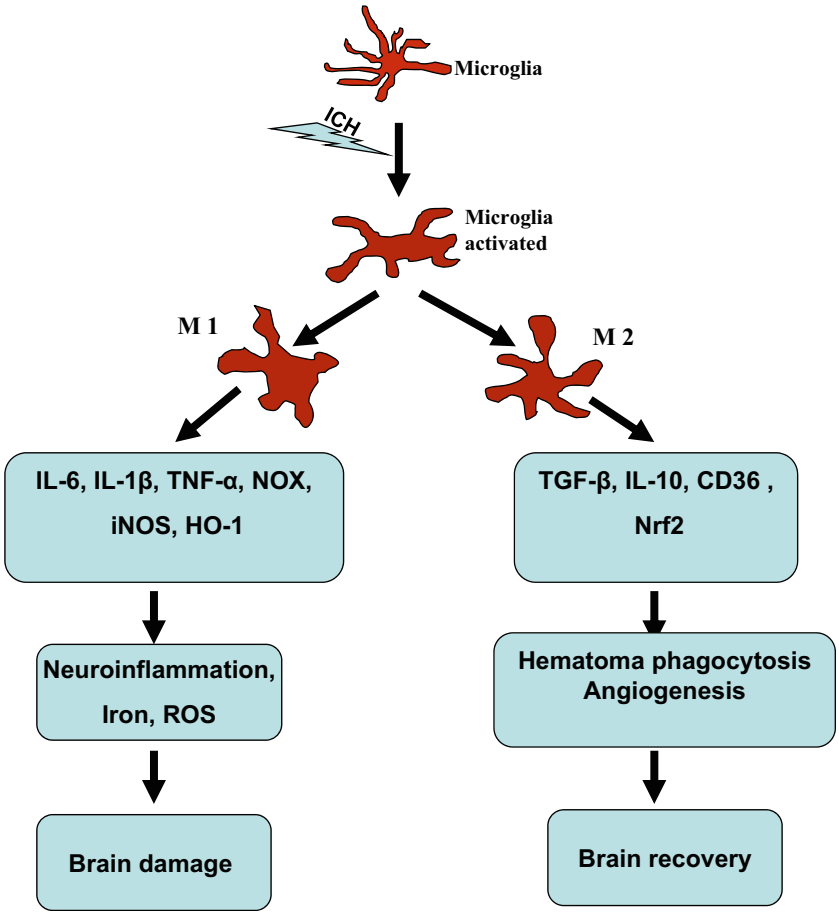

Fig. 1 After intracerebral hemorrhage (ICH), microglia can be activated/polarized to two phenotypes, M1 and M2. The M1 phenotype can produce proinflammatory mediators (interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α)), chemokines, redox molecules (NADPH oxidase (NOX), phagocyte oxidase (PHOX), inducible nitric oxide synthase (iNOS)), and heme oxygenase-1 (HO-1). These factors lead to neuroinflammation, iron accumulation, and reactive oxygen species (ROS) production and finally cause brain damage. The M2 microglia can produce anti-inflammatory mediators (IL-10, transforming growth factor beta (TGFβ)) and promote hematoma clearance through phagocytosis and promotion of angiogenesis. The Nrf2 signaling pathway may be involved in this process. Because they can be activated to either an M1 or M2 phenotype, microglia may have both proinflammatory and anti-inflammatory roles after ICH

microglia can also activate MMP-9, which mediates microglia-induced brain damage after ICH.

High-mobility group protein box-1 (HMGB1) is a highly conserved nonhistone DNA-binding protein that can be actively released into the cytoplasm and is involved in many inflammatory diseases [86–88]. Recent studies have shown a correlation between HMGB1 and acute ICH. In one study, HMGB1 was released from the nucleus into the cytoplasm in the ipsilateral brain as early as 1 h after ICH induction [89]. Moreover, ethyl pyruvate, which inhibits HMGB1 expression, significantly ameliorated ICH-induced neuroinflammation [89]. Similar effects were observed in ICH animals treated with glycyrrhizin (another nonspecific HMGB1 inhibitor) [86]. These findings suggest that HMGB1 can promote neuroinflammation after ICH. In the brain, many cells can express HMGB1, including microglia. In vitro, TNF-α stimulated cultured microglia to release large amounts of HMGB1 [90]. Recombinant human HMGB1 (rhHMGB1) also activated microglia, as demonstrated by increased NF-κB activity, increased production of NO, and upregulated transcription of cyclooxygenase (COX)-2, TNF-α, and IL-1β [91]. However, these effects were lost in $TLR4^{-/-}$ microglia treated with rhHMGB1 [91]. These observations suggest that HMGB1 can trigger microglial activation through the TLR4 receptor. Thus, microglial activation may be an important switch for HMGB1-mediated neuroinflammation after ICH. A recent study also has reported that long-term inhibition of HMGB1 reduced the recovery of neurologic function, decreased vascular endothelial growth factor and nerve growth factor levels in the ipsilateral striatum, and decreased the number of 5-bromo-2-deoxyuridine (BrdU)- and doublecortin-positive cells around the hematoma [92]. These results indicate that HMGB1 may promote angiogenesis and neurogenesis in the late phase of ICH. Thus, HMGB1-mediated neuroinflammation may be involved in angiogenesis and neurogenesis, suggesting a new therapeutic target for treating ICH.

Neuroprotection by Microglia After ICH

Although cumulative data suggest that inhibiting microglial activation could be beneficial to patients with ICH, long-term inhibition may be harmful because potentially neuroprotective functions of microglia, such as phagocytosis, could be inhibited [40]. The activation of microglia can promote hematoma and cell debris absorption and induce neurogenesis and angiogenesis. Several receptors on the surface of microglia are related to phagocytosis [93]. CD36, a class II scavenger receptor of microglia/macrophages, has been reported to bind to

phosphatidylserine, phosphatidylinositol, modified lipids, thrombospondin on sickle red blood cells, symmetric red cell ghosts, and apoptotic neutrophils to mediate phagocytosis. In vitro, transfection of CD36 into nonphagocytic cells confers the capacity for phagocytosis [94]. Inducing the expression of CD36 by peroxisome proliferator-activated receptor-γ (PPARγ) promotes hematoma absorption in the autologous blood injection model of ICH in mice [34]. Furthermore, a clinical study revealed that the absorption of hematoma is slower in $CD36^{-/-}$ ICH patients than in patients with normal CD36 expression [78]. Though the underlying mechanism by which CD36 promotes hematoma absorption needs additional investigation, promoting CD36 expression may represent a potential therapeutic option for ICH.

A recent study also has suggested that inflammatory factors can inhibit the expression of CD36. In $TLR4^{-/-}$ mice, CD36 expression is upregulated in perihematomal tissues after ICH [95]. Studies also have shown that inflammatory factors that are induced by TLR signaling, such as TNF-α, can regulate the expression of CD36 in macrophages [95, 96]. CD36 expression and hematoma absorption were significantly increased in $TLR4^{-/-}$ and $MyD88^{-/-}$ mice. What is more, the inflammatory factors TNF-α and IL-1β were induced by activation of microglia, and TLR4 signaling inhibited CD36 expression and hematoma absorption [78, 81]. These results suggest that TLR4 can regulate CD36-mediated hematoma absorption after ICH. Therefore, in addition to blocking the acute detrimental effects of microglial activation, stimulating microglial phagocytosis might offer therapeutic potential by enhancing recovery.

In addition to phagocytosis, microglia can promote neurogenesis and angiogenesis. Conditioned medium from microglia promotes neural precursor cell proliferation, migration, and differentiation [97]. When stimulated with IL-4, microglia promote insulin-like growth factor-1 expression, which causes neural precursor cell neurogenesis in a coculture system [55]. Some other factors secreted by microglia, such as protease serine 2, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor, can also promote neurogenesis [98]. Furthermore, microglia upregulate endothelial ephrin-A3 and ephrin-A4 expression to facilitate in vitro angiogenesis of brain endothelial cells [99]. These studies provide evidence that microglia may promote neurogenesis and angiogenesis, which are beneficial for brain function recovery.

Microglia-Correlated Inflammatory Mediators

Nrf2 is a transcriptional factor that regulates transcription of genes involved in antioxidative pathways, NADPH regeneration, and heme and iron metabolism [100]. It is regarded as a brain protector that can decrease microglial activation and oxidative injury after ICH [32, 101]. HO, which is downstream of Nrf2 signaling, can be activated by the heme released when hemoglobin is degraded. The HO isoforms (HO-1 and HO-2) then degrade heme to produce biliverdin, carbon monoxide, and iron. Increasing evidence suggests that accumulation of iron in brain can increase microglial activation and the production of free radicals that cause injury [26].

Nrf2

Recent genome-wide analysis showed that Nrf2 regulates hundreds of genes that are involved in the cytoprotective response against oxidative stress [102]. In normal, unstressed cells, Nrf2 is degraded by kelch-like ECH-associated protein 1 (Keap1). Keap1 can bind to the evolutionarily conserved N-terminal Neh2 regulatory domain of Nrf2 to suppress its transcriptional activity [102]. However, when cells experience oxidative stress, the cysteine residues in Keap1 are oxidized, leading to a conformational change that releases the suppression of Nrf2 activation [103]. In the brain, Nrf2 is expressed mainly in astrocytes, neurons, and microglia. It is thought to protect brain tissue from damage [104]. Accumulating evidence indicates that Nrf2 protects neurons from oxidative stress injuries after ischemic stroke in vivo and in vitro [105–107]. Compared with WT mice, $Nrf2^{-/-}$ mice suffered more brain damage in a cerebral ischemia model [107, 108]. In recent preclinical studies, sulforaphane [109], ursolic acid [110], erythropoietin [111], and (−)-epigallocatechin gallate [112] were shown to protect against brain damage after ischemic stroke by activating the Nrf2 pathway. However, Nrf2 has not been as well studied in ICH as it has been in ischemic stroke. We were the first to show that Nrf2 has a neuroprotective effect after the collagenase-induced ICH model [113]. Our study showed that ICH-induced brain damage is exacerbated in $Nrf2^{-/-}$ mice because of increased leukocyte infiltration and microglial activation (Fig. 2). This protective role of Nrf2 was confirmed by Zhao et al. [114] in an autologous blood model of ICH. A time course study showed that Nrf2 was significantly increased at 2 h and reached a peak at 24 h after ICH [115]. Treatment with Nrf2-inducer sulforaphane decreased the neutrophil count, oxidative damage, and behavioral deficits in WT mice but not in $Nrf2^{-/-}$ mice [114]. Additionally, (−)-epicatechin was shown to reduce early brain damage via synergistic Nrf2 pathways in collagenase and autologous blood models of ICH [32]. Although accumulating data suggest that Nrf2 has a neuroprotective role after ICH, the temporal and spatial distribution of Nrf2 is still unclear. Shang et al. [115] reported that Nrf2 is expressed chiefly in neuronal cells but not in glial cells. However, their assessment of Nrf2 distribution was limited to 2 h after ICH, whereas the peak of Nrf2 expression is at 24 h. Nevertheless, the activation of Nrf2 in microglia can induce antioxidative defense components, reduce peroxide formation, and upregulate CD36 expression

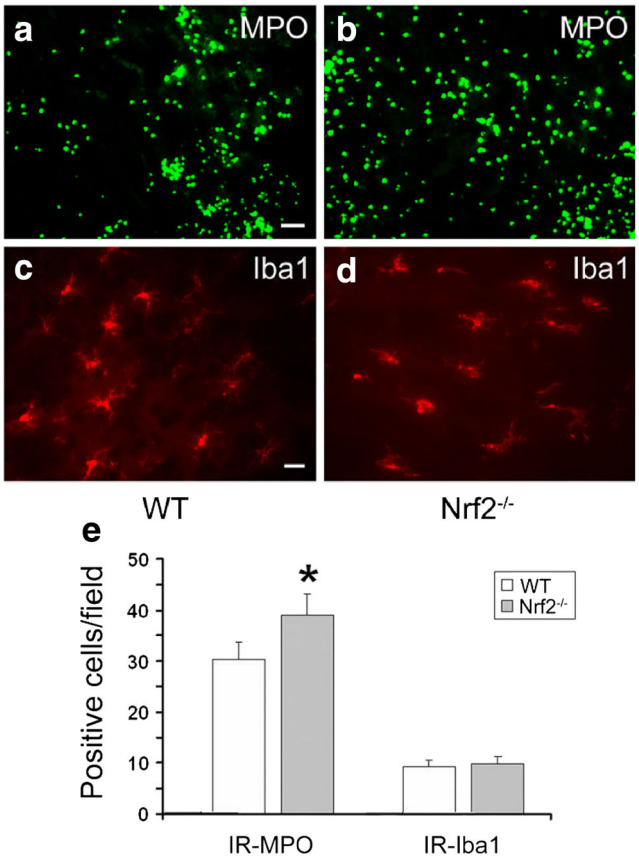

Fig. 2 Deletion of Nrf2 increases leukocyte infiltration but does not affect microglial activation in mice subjected to intracerebral hemorrhage (ICH). **a**–**d** Infiltrating neutrophils (MPO-positive cells; *scale bar* 40 μm) and activated microglia (Iba1-positive cells; *scale bar* 20 μm) were apparent in or around the injury site in Nrf2$^{-/-}$ and wild-type (WT) mice 24 h post-ICH. **e** Quantification analysis indicated that Nrf2$^{-/-}$ mice had significantly more infiltrating neutrophils than did WT mice at 24 h post-ICH; the number of activated microglia around the injury site was similar in Nrf2$^{-/-}$ and WT mice (both $n = 3$/group, $^{*}p < 0.05$) [113]

to enhance red blood cell phagocytosis in vitro [116]. The Nrf2 inducer sulforaphane can also induce CD36 expression to increase hematoma clearance in WT mice, but not Nrf2$^{-/-}$ mice, subjected to the autologous blood injection model of ICH [116]. Together, these data suggest that Nrf2 can regulate functional changes in microglia after ICH.

HO

After ICH, hemoglobin and its degradation products are highly toxic to brain tissue [77, 116]. Experimental evidence suggests that free heme liberated mainly from hemoglobin degradation contributes to the production of ROS and inflammation [117]. Because the extracellular free heme cannot be recycled in the brain, heme metabolism is critical to recovery after ICH. HO is the rate-limiting enzyme in the metabolism of heme to equimolar iron, carbon monoxide, and biliverdin, which is then converted to bilirubin by biliverdin reductase [118]. Two HO isoenzymes are present in mammalian cells: HO-1, the inducible isoform, and HO-2, the constitutive isoform. In the CNS, HO-1, also known as heat-shock protein 32 (HSP-32) [119], is expressed at a low level but can be rapidly induced by hemin [33], oxidative stress [120], heat stress [121], proinflammatory cytokines such as TNF-α and IL-1α [122], and anti-inflammatory cytokines such as IL-10 [123]. After ICH, HO-1 is induced mainly in microglia, whereas HO-2 is constitutively expressed in neuronal cells [26]. Though both HO-1 and HO-2 catalyze the same chemical reaction, their expression in different cell types suggests that they may have distinct roles during ICH.

The Effect of HO-2 After ICH

The unavailability of HO inhibitors has necessitated the use of genetically modified mice in mechanistic studies. HO-2$^{-/-}$ mice have been used to study the effect of HO-2 after ICH in autologous blood and collagenase models. However, the results in these two models differed. In the collagenase induced-ICH model, HO-2 deletion increased brain swelling, neuronal death, ROS, inflammation, injury volume, and neurologic deficits compared with those in WT mice [124, 125]. Moreover, HO-2 deletion did not appear to alter HO-1 expression from that in WT mice [124]. These data suggest that HO-2 may play a neuroprotective role in the collagenase-induced ICH model.

Whereas data from the collagenase-induced model suggested that HO-2 had a protective effect after ICH, data from the autologous blood induced-ICH model suggested a deleterious effect. Compared with outcomes in WT mice, HO-2 knockout attenuated perihematomal neuron loss at 4 and 8 days after blood injection and had a weak and variable effect on neurologic outcome [126]. Additionally, HO-2$^{-/-}$ mice exhibited a reduction in oxidative cell injury [127]. However, in this model, the expression of HO-1, which is thought to increase brain damage [26, 33], was significantly lower in the HO-2$^{-/-}$ mice than in the WT mice [127]. These distinct results may be caused by the different injury mechanisms in the collagenase and autologous blood injection models of ICH as well as the differences in HO-1 expression.

The Effect of HO-1 After ICH

Unlike the constitutive expression of HO-2, which accounts for most HO activity under normal conditions, HO-1 is expressed at a low level in normal brain and can be rapidly induced after ICH. Immunofluorescence shows that HO-1 is expressed in vascular-like structures [33] in the normal mouse brain (Fig. 3). HO-1 protein increases significantly after ICH and reaches a peak on day 3 [128]. We reported that HO-1 deficiency in mice reduced ICH-induced leucocyte infiltration, microglial/macrophage activation, and free radical levels

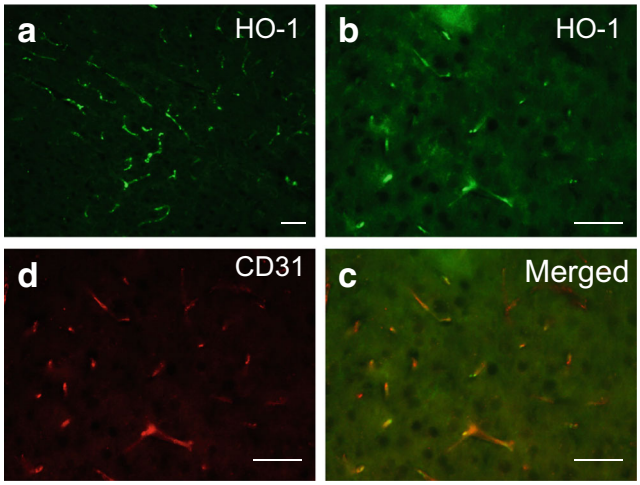

Fig. 3 Cellular localization of heme oxygenase-1 (HO-1) in normal mouse brain. **a** In normal mouse brain, HO-1 immunoreactivity was observed in vascular-like structures. **b**–**d** HO-1 immunoreactivity (*in green*) was colocalized with CD31-positive cells (*in red*, an endothelial marker). *Scale bar* 50 μm

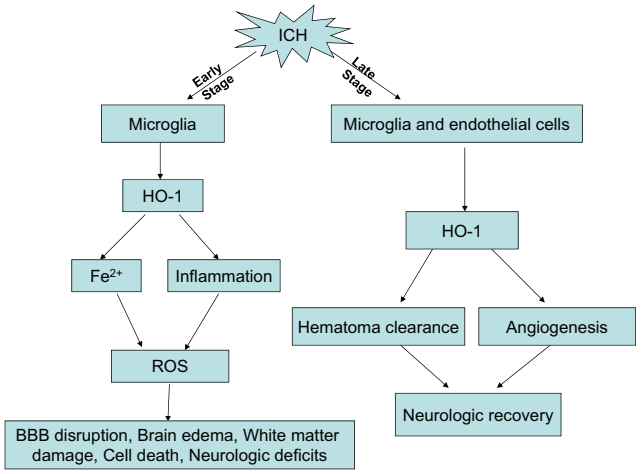

Fig. 4 In the early stage of intracerebral hemorrhage (ICH), activated microglia express high levels of heme oxygenase-1 (HO-1). The elevated HO-1 may affect cell function, promote iron accumulation, and increase inflammation. These changes result in high reactive oxygen species (ROS) production and an increase in brain damage (blood–brain barrier (BBB) disruption, brain edema, white matter injury, cell death, and neurologic deficits). In the late stage of ICH, high HO-1 expression in microglia and vascular endothelial cells may contribute to hematoma absorption and angiogenesis, thereby promoting neurologic recovery

compared with those in WT mice, but that brain water content did not change significantly [33]. These findings suggested that HO-1 increases brain damage in the early stage of collagenase-induced ICH in mice. No reports have described the use of HO-$1^{-/-}$ mice in the autologous blood-induced ICH model. Porphyrin HO inhibitors also have been used to study the role of HO-1 after ICH. These studies have consistently shown that porphyrin HO inhibitors are neuroprotective in the blood-injection ICH model [129, 130]. These findings suggest that HO-1 mediates brain injury after ICH and raise the possibility that HO-1 may be a therapeutic target for early-stage treatment of ICH.

HO-1 expression correlates significantly with the antioxidant copper–zinc superoxide dismutase (Cu/Zn-SOD) on days 1 to 3 after ICH, but no definitive correlation has been identified on days 7 and 14. Conversely, HO-1 correlates significantly with the prooxidant malondialdehyde on days 7 and 14 after ICH but not on days 1 to 3 [128]. Recent studies also have shown that hemin-induced HO-1 expression in perivascular cells before ICH can attenuate blood–brain barrier disruption after ICH [131]. These data suggest that HO-1 may have both antioxidative and oxidative roles in the pathophysiology of ICH (Fig. 4).

Iron

Iron, one of the heme (hemin) degradation products, can accumulate in the brain parenchyma for months after ICH and is very damaging [44, 132–135]. In the brain, microglia regulate iron homeostasis by sequestering and storing it within ferritin [136]. However, overaccumulation of iron can influence the physiologic properties of microglia [136, 137]. When exposed to lipopolysaccharide, iron-loaded primary microglial cells promote secretion of proinflammatory cytokines, such as TNF-α and IL-1β [136]. Furthermore, HO-1, the key enzyme for heme degradation, is mainly expressed in microglia after ICH. The high expression of HO-1 can also exacerbate brain damage by promoting microglial activation and iron deposition [32, 33].

Another potential mechanism by which iron causes brain damage is the generation of free radicals. In the Fenton/Haber-Weiss reaction, iron reacts with lipid hydroperoxides to produce free radicals that attack DNA and cause oxidative brain injury [135, 138, 139]. The excess iron in brain tissue after ICH can cause brain edema, neuronal death, brain atrophy, and poor neurologic outcomes [45, 135, 140]. Chelators can bind "free" iron to form a stable complex and prevent the iron from entering into the Fenton/Haber–Weiss reaction. Therefore, removing excess iron with iron chelators is a common practice. Many studies, including our own, have shown that deferoxamine, a ferric iron chelator, decreases microglial activation, oxidative stress, brain edema, and neuronal death and improves functional outcome after ICH [43, 44, 141–143]. However, others claim that although deferoxamine can reduce iron content in the brain, it cannot attenuate injury or improve neurologic function [144–146]. In one recent study, ferrous iron chelator (clioquinol), but not ferric iron chelator (deferiprone), improved brain outcomes after ICH [147]. Ferric iron chelator is more likely to facilitate the Fenton reaction process and increase free radical production. Though the use of iron chelators to reduce brain injury needs additional investigation before it can be applied clinically,

decreasing iron accumulation/toxicity in the brain remains an important goal for treatment of patients with ICH. Deferoxamine is now under phase II clinical trial for treating ICH patients [148].

Conclusion

The variable effects of microglial activation/polarization in models relevant to ICH suggest that they are challenging therapeutic targets. In the early stage of ICH, microglia can be activated by blood components and tend to acquire the M1 phenotype. M1 microglia express high levels of TLR4 and HO-1 to clear the hematoma, but they also increase inflammation, iron content, and free radical production, which exacerbate brain injury in the early stage after ICH. With hematoma resolution, the inflammatory reaction decreases and microglia change to an M2 phenotype, which promotes hematoma absorption and neurologic functional recovery. Inhibition of M1 microglial activation at the early stage of ICH and promotion of a shift from M1 to M2 phenotype in the late stage could be a potential therapeutic strategy for ICH treatment.

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