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Chapter 7

Recovery from ICH – Potential Targets

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1. Introduction

Intracerebral hemorrhage (ICH) is a devastating clinical event caused by rupture of blood vessels and accumulation of blood in the brain. Many disorders, including hypertensive arteriosclerosis, amyloid angiopathy, neoplasia, coagulation disorders and cerebrovascular malformations, directly or indirectly damage blood vessels in the brain and thus lead to ICH. The annual occurrence of ICH is estimated to be approximately 0.12 million in the USA and 2 million in the world. These numbers are expected to increase due to the aging of populations. Although accounting for only 15-20% of all strokes, ICH has severe clinical symptoms and poor prognosis. The 1-year survival rate of ICH is estimated to be 38% and long-term physical and mental disability is found in more than 90% of the survivors. Sadly, there is no effective treatment for ICH. Currently, primary supportive care and risk factor control are the main therapy for ICH in clinics. Thus, research and development of effective reagents to treat ICH is extremely urgent. In this chapter, we first introduce the anatomy and biology of the blood brain barrier. Then the pathophysiology and animal models of ICH are reviewed. Furthermore, we summarize the potential therapeutic targets for ICH.

2. Blood brain barrier

One unique feature about the blood vessels in the brain is the presence of the blood-brain barrier (BBB). BBB is a natural barrier that separates the central nervous system (CNS) from the circulation [1]. Under physiological conditions, the BBB prevents the entrance of blood cells and large molecules into the brain, but allows the uptake of nutrients and hormones from the blood, maintaining the homeostasis of CNS microenvironment [1, 2]. Under pathological conditions, the integrity of BBB is compromised and blood components leak into the brain, contributing to the progress of diseases [3-12]. At the cellular level, the BBB consists of brain

microvascular endothelial cells (BMECs), astrocytes, pericytes, neurons, microglia, and the non-cellular component-basement membrane [13] (Figure 1).

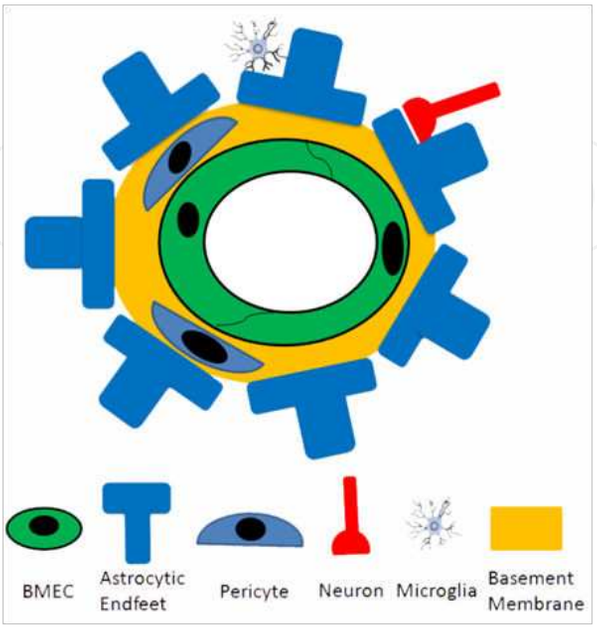

Figure 1. Schematic illustration of BBB. The BBB is composed of cellular and non-cellular components. Cellular components include BMECs, perycites, astrocytic endfeet, neurons, and microglia. Non-cellular components includes the basement membrane.

a. BMECs

Endothelial cells in the CNS, BMECs, are different in many ways from the ones in the periphery. First, BMECs have more mitochondria, lower pinocytotic activity, and little-to-no fenestrations. Second, the endothelium in the brain and the spinal cord is 50-100 times tighter than that in the rest of the body [14]. In the CNS, BMECs connect to each other via tight junctions, which are unique structures that confer impermeability to the BBB. Two types of proteins are found at tight junctions: transmembrane proteins, including occludin and claudins, and cytoplasmic accessory proteins, including zonula occluden-1, 2, 3 (ZO-1, 2, 3) and cingulin [15, 16]. The transmembrane proteins seal gaps between adjacent cells, decreasing intercellular permeability [17, 18], whereas cytoplasmic accessory proteins link transmembrane proteins to cortical actin-based cytoskeleton, enabling strict regulation of the distribution of tight junction proteins (TJP) [19, 20].

Besides intercellular transportation, intracellular transportation is another way to regulate BBB permeability [11, 21-23]. Although small lipophilic molecules, such as oxygen and carbon dioxide, can diffuse across BMECs freely [24], the transport of large hydrophilic molecules is mediated by specific transporters or receptors. Based on their subcellular distribution and functions, these transporters and receptors are divided into three groups. Group I transporters are expressed on both luminal and abluminal sides of BMECs and function to transport nutrients between the blood and brain [25, 26]. For example, glucose transporter 1 (GLUT1)

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transports glucose; monocarboxylate transporter 1 (MCT1) transports lactate; the L1 and y +transporters transport large neutral and cationic essential amino acids to and from the brain. Group II transporters are also expressed on both sides of BMECs, but only transport materials in one direction [27-29]. For example, transferrin and insulin receptors (TFR and IR) are expressed on both sides of BMECs. The luminal and abluminal receptors mediate endocytosis of transferrin and insulin from the blood and brain, respectively. Group III transporters are expressed on only one side of BMECs and usually mediate one-way transportation of materials [26, 30-38]. For instance, in order to remove excitatory neurotransmitter glutamate from the brain, excitatory amino acid transporters (EAATs) are exclusively expressed on the abluminal side of BMECs. Similarly, to facilitate the removal of amyloid-β from the brain, low-density lipoprotein receptor related protein 1 (LRP1) is solely expressed on the abluminal side of BMECs. Another example of such transporters is (Na^+-K^+) ATPase, which is only found on the abluminal side to regulate ion homeostasis and thus proper neuronal & synaptic functions. Additionally, multidrug resistance related protein 1 (MRP1) and P-glycoprotein (P-gp) are primarily expressed on BMEC luminal side to efflux many types of drugs from the brain. The subcellular distribution of these transporters and receptors is summarized in Figure 2.

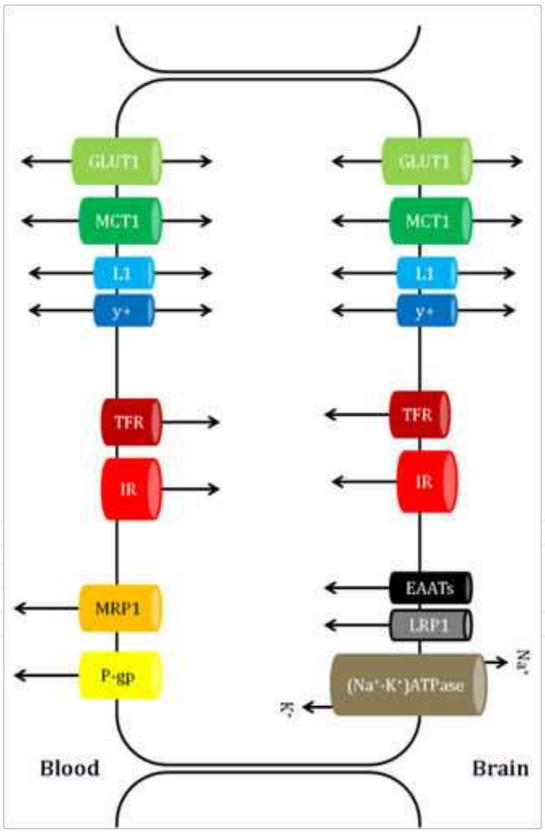

Figure 2. Major transporters and receptors expressed by BMECs. Three groups of transporters are expressed in BMECs. Group I includes GLUT1, MCT1, L1 and y+transporters, which are expressed on both luminal and abluminal sides of BMECs and transport materials bi-directionally. Group II includes TRP and IR, which are expressed on both sides of BMRCs but transport materials in one direction. Group III includes EAATs, LRP1, (Na+-K+)ATPase, MRP1 and P-gp, wich are expressed on only one side of BMECs.

b. Astrocytes

More than 30 years ago Stewart and Wiley, using xenograft experiments, demonstrated that the unique properties of BMECs, including increased mitochondria number, few pinocytotic vesicles and presence of tight junctions [39], were induced by the microenvironment of the CNS. Astrocytes, which constitute the major glial cells in the brain that cover more than 99% of the vascular surface using their extended endfeet [40, 41], have been suggested to contribute to these unique features of BMECs as well as the impermeability of BBB. Consistent with this hypothesis, temporary focal loss of astrocytes positively correlates with the compromise of BBB integrity *in vivo* [42]. Additionally, injected astrocytes have been shown to cover the blood vessels in the eye and prevent the leakage of Evans blue from the circulation system [43]. Moreover, *in vitro* culture experiments revealed that BMEC-astrocyte co-culture had a higher transendothelial electrical resistance (TEER) and less leakage of tracers, compared to BMEC monolayer [44-46]. Further mechanistic studies have demonstrated that both direct contact and astrocyte-secreted soluble factors, such as Ang1, TGF-β, GDNF and FGF2, are responsible for the impermeability of BBB [47-49]. These data suggest that astrocytes, by interacting with BMECs directly and indirectly, contribute to the unique properties of BMECs and the impermeability of BBB. Therefore, the co-culture of BMEC with astrocytes has been one of the most widely used *in vitro* BBB models, since it replicates in a petri dish the tight structures observed *in vivo*.

c. Pericytes

Discovered in 1873, pericytes are perivascular cells sandwiched between endothelial cells and astrocytic endfeet [50]. They are embedded in the basement membrane under normal conditions [1]. Pericytes cover capillaries and the degree of coverage varies depending on the species and tissue type[51]. It has been shown that the pericyte-to-endothelial ratio is 1:5 in rats, 1:4 in mice, and 1:3-4 in humans [52, 53]. In mice, this ratio is 1:1 in retina, 1:3 in brain and 1:100 in skeletal muscle vasculature [54], representing how tightly the blood vessels and their contents are confined in different tissues. Pericytes have several different developmental origins, depending on the organs they cover [51]. In the brain and thymus, pericytes arise from ectoderm-derived neural crest, whereas they differentiate from the mesothelium in the lungs, liver, and gut [51]. So far, there are no pericyte-specific markers available [51], although many cellular markers, including α-smooth muscle actin (SMA), PDGFRβ, Desmin, CD13, NG2, and RGS-5, have been used to identify pericytes, primarily in combination, as none of these markers is exclusive for these cells (pericytes share markers with myofibroblasts, vascular smooth muscle cells and neuronal progenitors [51]). It should be noted that the expression of these markers is high dependent on the differentiation stage of pericytes.

The main functions of pericytes include BBB regulation, vascular development and injury repair [52, 55, 56]. Here we focus on BBB regulation. It has been shown that pericyte-deficient mice have compromised BBB and pericyte coverage positively correlates with the tightness of tight junction [11, 57, 58]. Additionally, pericytes migrate away from capillaries, decreasing their coverage, under pathological conditions, such as hypoxia and traumatic brain injury [59, 60]. These data suggest that pericytes play a critical role in BBB integrity and maintenance.

Mechanistic studies demonstrate that BBB breakdown in pericyte-deficient mice is due to diminished expression of BBB-specific genes in endothelial cells and lack of polarity in astrocytic endfeet [58]. Consistent with these data, adding pericytes to BMEC-astrocyte co-culture system significantly enhanced TEER and decreased the leakage of tracers [61, 62]. Further studies showed that the function of pericytes on BBB integrity is also dependent on the differentiation stage of pericytes [63]. TGF-β treated pericytes, which are further differentiated SMA^+pericytes, compromise BBB integrity. On the contrary, b-FGF treated pericytes, which are less differentiated SMA^-pericytes, maintain impermeability of BBB. Altogether, these data suggest that pericytes is a key regulator of the BBB integrity. Nowadays, BMEC-pericyte-astrocyte triple-culture is becoming more and more popular in BBB research.

d. Neurons

In the human brain, the number of neurons and capillaries is estimated to be the same [64]. Both BMECs and astrocytic processes are directly innervated by noradrenergic, serotonergic, cholinergic, and GABA-ergic neurons [65-71]. The fact that local neuronal activity and metabolism regulate cerebral blood flow (neurovascular coupling) suggests that neurons may regulate BBB permeability through modulating BMEC and astrocyte function [72]. Consistent with these data, adding neurons to *in vitro* BBB models significantly increases the tightness of the BBB [73]. However, the exact mechanism underlying how neurons contribute to the BBB integrity is still elusive. Many studies focus on such mechanisms.

e. Microglia

Microglia, the brain resident immune competent cells, account for 10-20% of glial cells in the brain [74, 75]. Fate mapping studies suggest that they originate from Myb-independent, FLT3-independent, but PU.1-dependent myeloid progenitors that express colony stimulating factor 1 receptor (Csf1R) at embryonic day 8.5 [76-80]. Under physiological conditions, microglia have a ramified morphology, characterized by a small cell body and many long/thin dynamic processes [75]. By extending and retracting these dynamic processes, microglia survey the changes of microenvironment in the brain [75]. Once an insult is identified, microglia quickly undergo a process collectively termed activation, which involves changes to ameboid morphology. Activated microglia migrate to the site of injury, proliferate locally, secrete pro-and anti-inflammatory cytokines, and remove cellular debris by phagocytosis [74, 81-83]. Microglia play a dual role in the brain. On one hand, they contribute to neurite growth and neuronal survival by clearing cell debris and releasing neurotrophic factors [84-86], such as neurotrophin-3 and brain-derived neurotrophic factor. On the other hand, microglia secrete high levels of pro-inflammatory cytokines, including TNF-α and IL-1β, promoting neuronal death. The former (neuroprotective microglia) display anti-inflammatory properties and are called M2 cells, similar to the nomenclature of macrophages. The latter, secreting pro-inflammatory cytokines, exhibit neurotoxic behaviors and are called M1 microglia. Which role they play is highly dependent on the timing after injury and the type of injury. Since microglia are close to other components of the BBB in the brain, they may regulate BBB integrity either by directly interacting with the blood vessels, or indirectly through interaction with BMECs, astrocyte endfeet, or pericytes [87]. Interestingly, microglial activation has been reported to both

compromise and restore BBB integrity [88, 89]. This discrepancy could be explained by different injury models and different timing after injury. More work is needed to answer the question how microglia regulate BBB integrity.

f. Basement Membrane (BM)

BM is a 3-dimensional network composed of extracellular matrix (ECM) proteins, including collagens, laminins, heparin sulfate proteoglycans, and nidogens [47, 90]. The formation of this network involves polymerization and cross-link of these ECM proteins [90, 91]. At the BBB, BMECs generate a vascular BM and astrocytes generate a parenchymal BM [92, 93]. The vascular and parenchymal BM is usually indistinguishable at capillaries [1]. However, at the post-capillary venules, the two BMs are separated by perivascular space where cerebrospinal fluid drains, and where antigen-presenting cells can be found [1]. Both BM layers have the same composition except that in the vascular BM laminin-α4 and-α5 are predominantly present [93], whereas in the parenchymal BM laminins-α1 and-α2 are the main components [92-94].

Accumulating evidence suggests that loss of BM results in disruption of BBB, probably due to the loss of a physical barrier at the BMEC-astrocyte interface and/or lack of signaling from ECM molecules [95-99]. Individual ECM proteins, including laminin, collagen type IV, and fibronectin, have been shown to increase the TEER of BMECs *in vitro* [100]. Using laminin conditional knockout mice, we have shown that astrocytic laminin maintains BBB integrity by preventing pericyte differentiation from the resting stage to the contractile stage [101]. In addition, laminin α5 and dystroglycan, a major receptor for ECM proteins, have been found to negatively correlate with the infiltration of leukocytes in the brain [93]. These data suggest that BM plays a crucial role in BBB regulation. Future studies are expected to focus on the roles of individual ECM proteins in BBB integrity. Understanding how these ECM proteins affect individual BBB components and BBB integrity would significantly enhance our knowledge on BBB and potentially pave the way for the treatment of many neurological disorders.

3. Pathophysiology

When ICH occurs, blood leaks into the brain parenchyma, leading to the formation of hematoma, which quickly increases intracranial pressure. The accumulated blood and high intracranial pressure cause immediate primary damage to the brain. This initial injury is followed by secondary damage mainly resulting from inflammatory responses [102, 103]. The exposure of brain parenchyma to blood proteins (e.g., proteases and hemoglobin) and cells (red blood cells and leukocytes) results in activation of microglia, and the secretion of pro-inflammatory cytokines/chemokines [104, 105], including TNF-α, IL-1β, and MCP1/CCL2. These inflammatory mediators, by forming a concentration gradient, activate and attract more microglia and other inflammatory cells to the injury site [106]. These cells then accumulate around the hematoma, forming a barrier to prevent the spread of injury to other sites. The released pro-inflammatory cytokines/chemokines and possibly activated microglia also act on BMECs, pericytes and astrocytes, leading to compromise of BBB integrity. Through the

disrupted BBB, peripheral leukocytes infiltrate into the brain. The infiltrated leukocytes together with activated microglia produce more pro-inflammatory mediators, which induce cell death in the penumbra area [107, 108]. In addition, hemolysis of red blood cells causes iron deposition in the brain parenchyma and subsequent lipid peroxidation [109]. Free radicals generated during lipid peroxidation also lead to cell death and contribute to ICH-induced brain injury [110, 111]. With the progress of disease, microglia and infiltrated leukocytes change their gene expression profile from pro-inflammatory to anti-inflammatory and clear up the dead cells via phagocytosis [110, 112]. The clearance of cell debris finally leads to the resolution of the hematoma and repair of damaged tissue. At this stage, the activated inflammatory cells revert to a resting state again. Due to the limited regenerative ability of neurons, however, most neurological functions cannot be recovered, which explains the high extent of disability after ICH.

4. ICH animal models

To study ICH and eventually cure this disease, several ICH animal models have been developed, including collagenase ICH model, whole blood ICH model, and the spontaneous ICH model. Although these models have been widely used in ICH research, none of them fully replicates the pathology of ICH in human patients. Here we briefly discuss the advantages and disadvantages of these models.

a. Collagenase ICH Model

This model utilizes the enzymatic activity of collagenase, a bacterial enzyme. After injection into the brain, collagenase induces rupture of blood vessels by degrading collagen IV, a component of the blood vessel wall [103-105]. The rupture of blood vessels then induces the formation of hematoma and other pathological alterations. There are many advantages of this model. First, ICH induced by collagenase injection is very reliable and reproducible. The size and location of hematoma reported by different laboratories across the world are comparable [112-115]. Second, the location of hematoma can be controlled depending on the site of injection. Third, this model is very simple and fast. ICH can be induced within hours after collagenase injection. Due to these advantages, collagenase ICH model has become one of the most popular animal models for ICH research. This model, however, also has a few disadvantages. One of the most significant drawbacks is that it introduces collagenase, a bacterial enzyme, into the mammalian brain. This enzyme degrades ECM proteins in the brain, affects BBB integrity, and modifies inflammatory or immune responses, all of which may affect ICH progress [105, 116, 117]. Another disadvantage of this model is that it does not replicate the vascular challenges usually seen before the onset of ICH in patients, such as hypertension and atherosclerosis. Mice lacking these vascular injuries may have different disease progress and/or recovery patterns, which makes it difficult to interpret data generated using this ICH model.

b. Whole Blood ICH Model

The whole blood model involves injection of blood from the same animal or a donor into the brain. The injected blood induces secondary pathological changes observed in human patients. Unlike the collagenase ICH model, this model does not introduce exogenous enzymes. The application of this ICH model, however, is circumvented by its three major disadvantages. First, the whole blood ICH model lacks pathological changes in blood vessels. The vascular challenges and rupture of vasculature cannot be replicated in this model. Second, this model is less reproducible than collagenase ICH model. The size and location of hematoma vary depending on different laboratories. Third, the shape of hematoma is different from that found in human patients. Hematoma formed in whole blood ICH model is usually umbrella-shaped and narrower slit-like [118]. This unique shape is probably caused by high pressure-induced rapid distribution of blood along white matter tracts and/or corpus callosum after injection. A way to get around this problem is used in bigger animals, like pigs, where a space/balloon forming initial injection is followed by the injection of the homologous whole blood.

c. Spontaneous ICH Model

To better replicate the pathological changes observed in human patients, a spontaneous ICH model has been developed in rodents [119]. This new model induces ICH through acute hypertension, the most common etiology of hemorrhage in humans. In this model, animals are administered with NG-nitro-L-arginine methyl ester (L-NAME) and angiotensin II to induce hypertension. The injection of angiotensin II causes surges of blood pressure, which eventually lead to rupture of blood vessels and thus ICH. This spontaneous ICH model replicates most pathological alterations observed in human patients. However, the time it takes to induce ICH is relatively long (2-4 weeks), the location of the ICH varies, and the reproducibility still needs further investigation.

5. Targets for ICH treatments

ICH is a devastating clinical event. Sadly, no effective treatments are available at present. Current therapy is mainly supportive care [120, 121]. Due to the pivotal role of inflammatory responses in ICH development, anti-inflammatory strategies have been explored by many laboratories. Here we review a few anti-inflammatory targets with therapeutic potential in ICH: microglial activation, leukocyte infiltration, cytokines/chemokines, protease activation, and reactive oxygen species (ROS) production. In addition, stem cell therapy is also discussed briefly.

a. Microglial Activation

Microglia are one of the first cell types that respond to ICH. In collagenase ICH model, microglial activation starts at 1 hour [102, 122], peaks at 3-7 days [104, 105, 115, 123], and returns to a resting state again by 3-4 weeks after the onset of ICH [124, 125]. A similar time course of microglial activation is observed in whole blood ICH model [122, 124, 125]. Since activated microglia contribute to the amplification of inflammatory responses and cell death by secreting chemotactic cytokines and cytotoxic mediators, including proteases and ROS [102, 103, 112,

115], inhibition of microglial activation has been proposed as a therapeutic strategy for ICH. It has been shown that pre-or post-treatment with the tri-peptide microglia/macrophage inhibitory factor (MIF, Thr-Lys-Pro) significantly inhibited microglial activation, reduced injury size and improved neurological function [104, 105]. Consistently with this report, inhibiting microglial activation with neuroprotectant minocycline in both collagenase and whole blood ICH models protected BBB integrity, decreased brain edema, and improved functional recovery, although neuronal death remained changed [126-130]. These data support that inhibition of microglial activation is beneficial. However, there is also evidence suggesting that long-term inhibition of microglial activation is detrimental [104, 115]. Given that activated microglia also contribute to the clearance of cell debris and recovery at late stage, inhibition of microglial activation should be limited to the early stage. The question then becomes how to define early and late stages after ICH? Definition of these stages would significantly improve the outcome of ICH treatments.

b. Leukocyte Infiltration

Leukocytes infiltrate into the brain through the compromised BBB and modulate the progress and/or recovery of ICH [102, 112]. Among all the subtypes of leukocytes, neutrophils are the earliest ones to infiltrate into the brain after ICH. In both collagenase and whole blood ICH models in rodents, neutrophil infiltration starts at approximately 4 hours and peaks at 3 days after the onset of ICH [102, 115, 124, 131, 132]. These cells promote cell death and brain damage by producing ROS and pro-inflammatory mediators [107, 108], and usually die within 2 days in the brain. Mice deficient for CD18, a subunit of β2 integrin indispensable for leukocyte infiltration, demonstrated reduced brain edema and mortality as well as decreased leukocyte number in the brain after collagenase injection [133]. In human postmortem brains, leukocyte infiltration was also observed within hours after ICH [134, 135]. Furthermore, leukocyte counts in blood have been found to positively correlate with injury size in ICH patients [136]. Therefore, high leukocyte counts together with other factors have been used to predict early clinical outcome in ICH patients [137, 138]. Currently, no anti-leukocyte infiltration strategies have been investigated in ICH models. Obtaining such data may facilitate the research and development of novel reagents targeting leukocyte infiltration.

c. Cytokines/Chemokines

During ICH, activated microglia and infiltrated leukocytes produce high levels of inflammatory cytokines/chemokines, which mediate the secondary damage to the brain. In both rodents and humans, pro-inflammatory cytokines, including TNF-α and IL-1β, are transiently up-regulated in the peri-hematomal region [106, 139]. In addition, chemokines and chemokine receptors that mediate leukocyte extravasation, including CCL2-4, IL-8, CXCL5, and CCR1-2, are also increased/activated [139, 140]. These data suggest that targeting cytokine/chemokine signaling may be a therapeutic strategy for ICH. In collagenase ICH model, we have found that mice deficient for CCL2 or its receptor CCR2 have a mild but delayed disease progression [115]. In $CCL2^{-/-}$or $CCR2^{-/-}$mice, hematoma was smaller at day 1 post injury (dpi 1) but larger at subsequent times (dpi 7 and 14 [115]), indicating a delayed recovery. Consistent with the crucial role of CCL2-CCR2 system in microglial activation/migration, limited numbers of

microglia were observed at dpi 1 in both knockout mice [115]. At dpi 3 and 7, however, the number of microglia in the knockout mice far exceeded those in control animals [115], suggesting that CCL2-CCR2 independent alternative signaling recruited microglia in the knockout mice. The infiltration of neutrophils was also ablated in both knockout mice at dpi 1 and 3, echoed by the smaller hematoma size early after injury [115]. In addition, at dpi 7 the expression of inducible nitric oxide synthase (iNOS) decreased in controls compared to earlier time-points, but remained high in the mutant mice, indicating that lack of CCL2-CCR2 signaling produces more ROS. Moreover, brain edema, neuronal loss and neurological function followed similar trends over time as that of hematoma size [115]. Altogether, these data suggest that inhibiting CCL2-CCR2 signaling early after ICH is neuroprotective, whereas long-term inhibition delays the recovery. Future work should focus on developing the best CCL2-CCR2 inhibition regimen for ICH patients.

d. Protease Activation

ICH activates many proteases, including matrix metalloproteinases (MMPs). MMPs are a group of zinc-dependent proteases actively involved in extracellular remodeling and neuro-inflammation. Under physiological conditions, low levels of inactive MMPs are found in the brain. These MMPs, however, are dramatically up-regulated and activated when ICH occurs [112, 141]. We and others have demonstrated that collagenase quickly activates and up-regulates the expression of MMP-2,-3,-9, and-12 in rodents [112, 142]. Activation of MMP-9 has also been described in other ICH models [143-145]. In human ICH patients, blood MMP-9 level has been reported to correlate with BBB integrity, hematoma size, edema of the penumbra area, and neurological function [138, 146, 147], whereas blood MMP-3 levels have been found to associate with mortality [148]. Additionally, higher level of MMP-9 was detected in the peri-hematomal region in postmortem human brains [113, 149]. These data suggest that modulation of MMP activity may have therapeutic effect in ICH. Consistent with this hypothesis, mice lacking MMP-3,-9, or-12 are partially protected from ICH [141, 144, 150]. In addition, the therapeutic effect of MMP inhibitors has also been investigated. GM6001, a broad-spectrum MMP inhibitor, has been found to be neuroprotective in both collagenase and whole blood ICH models in mice [132, 151]. Similar results have been noted for BB-1101, another broad-spectrum MMP inhibitor [152]. However, both neuroprotective and detrimental roles have been reported for MMP inhibitor BB-94, depending on the animal models used [153-155]. Besides its inhibitory effect on microglial activation, minocycline also functions as a MMP inhibitor [126]. There is evidence suggesting that minocycline reduces TNF-α level and brain edema without affecting neuronal loss [127, 156], when administered 6 hours after ICH. Together, these data suggest that MMPs, especially MMP-9, play a detrimental role in ICH, and that MMP inhibitors may be used, alone or in combination with other medicine, to treat ICH.

e. ROS Production

One of the main pathological changes of ICH is the accumulation of blood in the brain. The hemolysis of extravasated red blood cells leads to degradation of hemoglobin and deposition of iron in the brain [109]. In rats, a 3-fold increase of non-heme iron was found after ICH [109].

Accumulated iron has been shown to induce oxidative stress by formation of free radicals, mediate secondary inflammatory injury, and contribute to brain atrophy and neurological deficits after ICH [157, 158]. In human patients with spontaneous ICH, blood ferritin level associates with brain edema in peri-hematomal region [159]. In addition, iron level in the hematoma also correlates with brain edema in peri-hematomal area [160]. These data suggest that iron deposition contributes to brain damage, and that removing the deposited iron may be an appropriate therapeutic approach. Consistent with this hypothesis, 2, 2'-dipyridyl, a lipid-soluble iron chelator, has been shown to be beneficial in both the collagenase and whole blood ICH models in mice [161]. Another iron chelator deferoxamine has shown neuroprotective effects in the whole blood ICH model in rats and piglets [162-165]. In collagenase ICH model, however, deferoxamine failed to show any beneficial effects [166], suggesting the effect of deferoxamine depends on ICH animal models. None-the-less high doses of deferoxamine are currently examined in clinical trials (starting in 2012) for the treatment of ICH.

An alternative way to treat iron-induced oxidative stress is to target antioxidant enzymes. To remove extra ROS, antioxidant enzymes, including glutathione S transferases, glutathione peroxidase, and glutamate-cysteine ligase, are up-regulated. The key transcription factor that controls the expression of these antioxidant enzymes is Nrf2 [167]. Nrf2 is expressed in neuronal and glial cells in the brain. Activation of Nrf2 has been shown to be neuroprotective both *in vitro* and *in vivo* [168, 169]. Additionally, mice deficient for Nrf2 showed more severe neurological deficits compared to wild-type mice in both collagenase and whole blood ICH models [170, 171]. Paralleled with neurological deficits, enhanced ROS production and leukocyte infiltration were observed in $Nrf2^{-/-}$mice [170, 171]. More importantly, sulforaphane, an Nrf2 inducer, has been reported to improve neurological deficits in mice when administered 30 minutes after ICH [170]. Together, these data suggest that Nrf2 is a target with therapeutic potential.

f. Stem Cell Therapy

ICH induces neuronal death and loss of neurological function. Multipotent stem cells with the ability to differentiate into neurons are a potential therapy for ICH. It has been reported that human neural stem cells are able to differentiate into neurons and astrocytes, and thus improve neurological function after intravenous injection in collagenase ICH model [172]. Stem cell therapy is relatively new and more work is needed before it can be used in ICH patients. For example, the route, dose and timing of stem cell injection need to be optimized; the differentiation, proliferation and integration of stem cells *in vivo* should be investigated; and the side effects of stem cell administration must be examined.

6. Summary

Accumulating evidence suggests that the secondary inflammatory responses play a critical role in the development of ICH, indicating that the molecular mechanism of inflammation is an ideal target for the therapy of ICH. As discussed, many pathways, including microglia activation, leukocyte infiltration, cytokine/chemokine secretion, protease activation, and ROS

production, have been explored, and several compounds showed significant potential in the treatment of ICH. However, it should be noted that the animal models used in the studies are not perfect, which limits the interpretation of experimental data. Thus, other models and human samples should be used to confirm the results before they are used in patients.

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