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REVIEW ARTICLE

Programmed Cell Death after Intracerebral Hemorrhage

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Abstract: ***Background*:** Intracerebral hemorrhage (ICH) accounts for up to 15% of all strokes and is characterized by high rates of mortality and morbidity. The post-ICH brain injury can be distinguished in 1) primary, which are caused by disruption and mechanical deformation of brain tissue due to hematoma growth and 2) secondary, which are induced by microglia activation, mitochondrial dysfunction, neurotransmitter and inflammatory mediator release. Although these events typically lead to necrosis, the occurrence of programmed cell death has also been reported after ICH.

***Methods*:** We reviewed recent publications describing advance in pre- and clinic ICH research.

***Results*:** At present, treatment of ICH patients is based on oral anticoagulant reversal, management of blood pressure and other medical complications. Several pre-clinical studies showed promising results and demonstrated that anti-oxidative and anti-inflammatory treatments reduced neuronal cell death, however, to date, all of these attempts have failed in randomized controlled clinical trials. Yet, the time frame of administration may be crucial in translation from animal to clinical studies. Furthermore, the latest pre-clinical research points toward the existence of other, apoptosis-unrelated forms kinds of programmed cell death.

***Conclusion*:** Our review summarizes current knowledge of pathways leading to programmed cell death after ICH in addition to data from clinical trials. Some of the pre-clinical results have not yet demonstrated clinical confirmation, however they significantly contribute to our understanding of post-ICH pathology and can contribute to development of new therapeutic approaches, decreasing mortality and improving ICH patients' quality of life.

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1. INTRODUCTION

Intracerebral hemorrhage, a pathological accumulation of blood in brain parenchyma, results from sudden blood vessel rupture. ICH accounts for only 10% - 15% of all strokes, however, it is associated with high mortality and morbidity [1]. Forty percent of ICH patients will die during the first month after ICH, and 75% of surviving patients will have long-time lasting neurological deficits [2]. The high rate of mortality and morbidity is a consequence of the lack of effective therapeutic options. Standard ICH management primarily focuses on supportive therapies and on oral anticoagulant reversal, management of blood and intracranial pressure, and maintenance of hemodynamic stability. Surgical treatment is essential in patients with obstructive hydrocephalus, brainstem compression as well cerebellar hemorrhage with neurological deterioration [3]. Trials investigating other therapeutic options failed so far [3]. Due to increasing life expectancy and aging of the population in the developed countries, the incidence of ICH will most likely escalate in the future. Therefore a better understanding of the ICH pathology, leading to the identification of the new therapeutic targets and consequently to the development of the new therapeutic approaches is urgently needed.

There are numerous factors that trigger post-ICH pathophysiological pathways leading to cell death in the *perihematomal* and remote brain regions. The neuronal cell loss is responsible for significant patient mortality and morbidity after ICH. In this review, we will summarize our knowledge with regard to ICH-induced cell death with focus on programmed cell death. Programmed cell death (PCD) characterizes any form of cell death caused by an intracellular death program including apoptosis, autophagy and pyroptosis [4] (Fig. **1**). Furthermore, we will discuss pre-clinical and clinical study results, which describe strategies leading to attenuation of post-ICH cell deaths, which might pave the way to new treatment strategies, a decrease in mortality, and improvement of the quality of life for ICH survivors.

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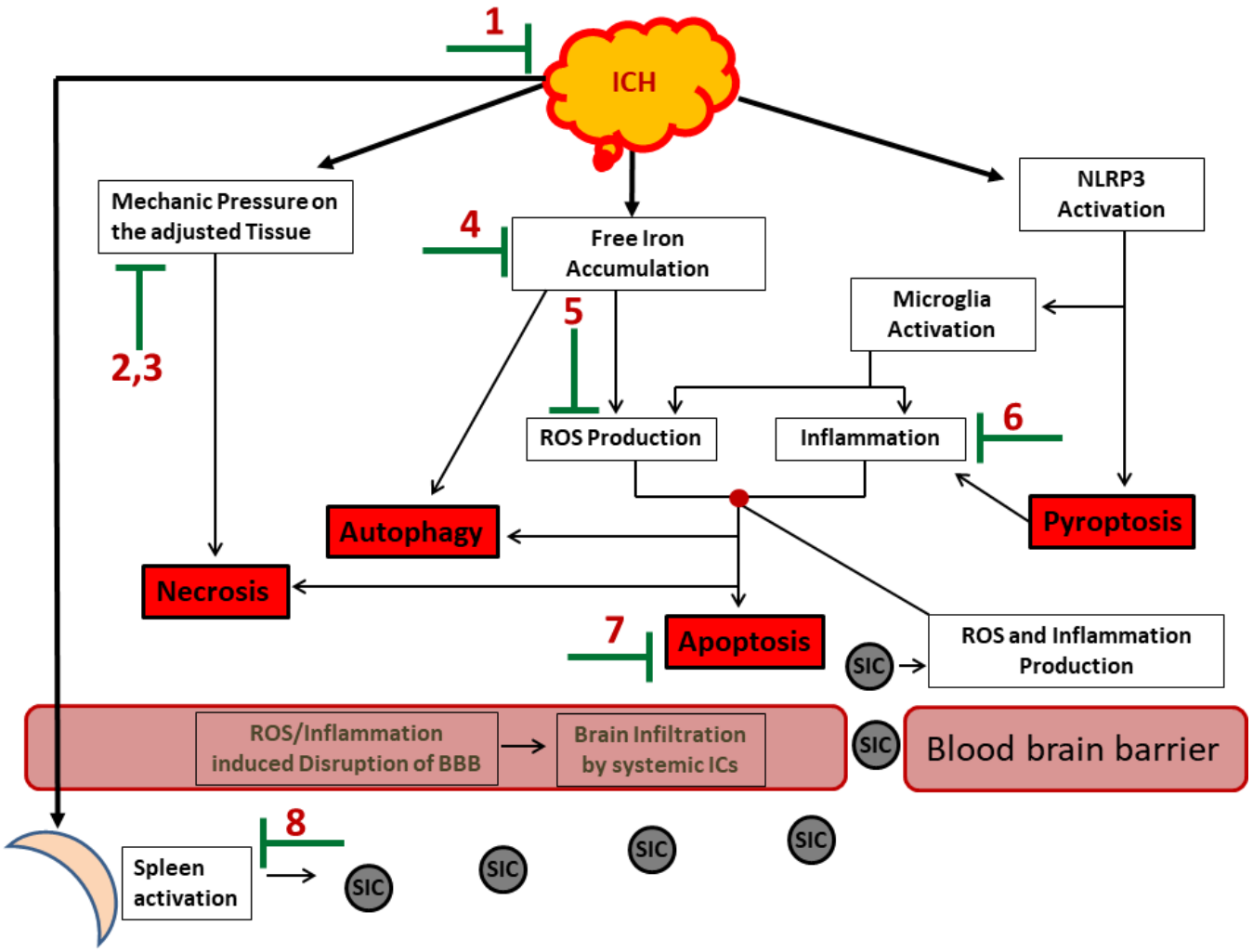

Fig. (1). Schematic presentation of major pathways leading to the programed cell death and clinical relevant interventions improving cell survival after ICH. Attenuation of programed cell death *via*: **1** Acceleration of hematoma clearance and CD36 mediated phagocytosis (PPARγ-agonists). **2** Surgical evacuation of hematoma. **3** Decrease of hematoma size *via* aggressive blood pressure reductions (INTERACT-2 and ATACH trials). **4** Free iron scavenging (deferoxamine Hi-Def (NCT NCT01662895) trial). **5** ROS scavenging (Minocycline, MACH trial; Edaravone, disufenton sodium (NXY-059), CHANT-trial). **6** Anti-inflammatory approaches (*e.g.* COX-2 inhibitors [celecoxib]). **7** Direct targeting of apoptosis (G-CSF). **8** Attenuation of post-ICH release of SICs and consequently brain infiltration by SIC (FTY-720).

2. CELL DEATH AFTER ICH

2.1. Necrosis

Necrosis is considered disorganized cell death. Necrosis does not occur spontaneously. It is considered a response to atypical conditions such as trauma or bleeding [5]. Necrosis results in cell swelling and lysis, leading to cell death with a subsequent and significant inflammatory response. Cell death has been well documented after ICH. Several studies demonstrated the presence of necrotic cells in ICH animal models. Qureshi *et al*. induced ICH in rabbits *via* infusion of 0.4 ml of autologous arterial blood (given the fact, that the size of the rabbit brain is approximately 1% of the human size; this volume mimicked the effects of 40 ml hematoma in the human brain [6]). Authors reported that 24 h after ICH induction significant numbers of necrotic cells were observed in the hematoma area. A high frequency of necrotic cells was observed in the ipsilateral cortex. Interesting enough, cells with necrotic appearance were also noticed in the contralateral cortex, indicating that the direct effects of blood pressure on the adjustment tissue is not the only factor involved in triggering post-ICH necrosis [7]. Authors were unable to detect neurological dysfunctions in this model. Apparently, necrosis can be induced even by a small damage and not necessarily lead to the development of neurological dysfunctions.

Extensive effects of hematoma on post-ICH necrosis were investigated by another group [8]. These authors used a "balloon" model of ICH in rats and induced ICH-like damage in a time-dependent manner by inflation of a micro-balloon in the brain of the animals for 10 or 120 min. Necrotic neurons were present in the lesioned areas. In the inner boundary lesion, necrotic neurons were also mixed with normal undamaged neurons. The authors showed that the lesion volume increases, depending on the balloon inflation time and that the lesion progressively increases after balloon

deflation. However, the correlation between lesion area and number of necrotic neurons was not calculated. Furthermore, the authors of this publication did not test neurological function in these animals. Correlation between neurological dysfunction and number of necrotic cells could have contributed to the understanding of the importance of ICH-induced necrosis. It is also worth to mention that the "balloon" model, used in that study, mimics the extensive effects of only the hematoma. It was, however, unable to reproduce all other factors leading to the post-ICH brain damage and did not completely mimic human ICH. Despite some shortcomings, this study clearly demonstrated that even a short time period can influence mechanical forces that inevitably leads to neuronal necrosis and also can result in brain damage after ICH.

The results of the study are in agreement with clinical studies. Qureshi *et al*. retrospectively investigated samples from 12 ICH patients after surgical hematoma evacuation and reported that 25% of cells in the perihematomal region underwent necrosis [6]. The number of necrotic cells caused by ICH varied from 0% to 100% and the presence of necrotic cells depended on the hematoma's location [6]. Furthermore, similar to the animal studies, the number of the necrotic cells depended on the time for hematoma evacuation. A maximum number of necrotic cells were observed in the patient, who underwent late hematoma evacuation (five days after ICH). In contrast, patients with early evacuation (< 24 h) presented with only marginal numbers of necrotic cells.

2.2. Apoptosis

While necrosis is considered non-programmed cell death, cell death can occur in a programmed manner. One example of programmed cell deaths is apoptosis. The morphological hallmarks of apoptosis include cell shrinkage and membrane blebbing with no organell changes. In apoptotic cells, chromatin condenses against the nuclear envelope followed by formation of pyknotic cell nuclei with cleaved DNA fragments. Then cell content is packaged into apoptotic bodies. The apoptotic cell separates from the surrounding tissue and will be rapidly phagocytosed into neighboring cells, including macrophages and parenchymal cells.

Two major pathways lead to apoptosis: 1) extrinsic and 2) intrinsic. The extrinsic cell death pathway requires cell surface receptors. Upon binding to their ligands, receptors activate caspase-8 and consequently its´ downstreams effectors [9].

The intrinsic apoptotic pathway is initiated by mitochondrial outer membrane permeabilization (MOMP) and a key member of the B-cell lymphoma 2 (Bcl-2) family. Bax, a member of the Bcl-2 family, promotes apoptosis by transferring from the cytosol to the mitochondrial outer membrane. Another member of the same family, Bcl-2, inhibits the apoptotic pathway. The increase in the proportion of Bax/Bcl-2 facilitates cell apoptosis [10].

The caspase-3, a member of the caspase family, is a key mediator of neuronal apoptosis and can be activated by both pathways [10].

Interestingly, the same stimuli (such as glutamate, thrombin, and reactive oxygen species [ROS]) responsible for induction of necrosis are also considered apoptotic inductors [11-13]. It is possible that as a result of the similarity of initial stimulus, a mixture of necrotic and apoptotic cells is present in the brain after ICH. Qureshi *et al*. observed a high frequency of both apoptosis and necrosis in the cells in the rabbit brains after autologous blood injection [7]. Similarly, Nakashima *et al.* (whose publication was mentioned in the "necrosis" section) demonstrated a time-dependent increase of apoptotic cells in the ICH-"ballon" model. The number of apoptotic cells became significantly elevated after 6 hours and peaked 24-48 hours after damage [8]. Both working groups used TUNEL staining for the visualization of apoptotic cells. The staining is considered the "gold standard", however, later it was demonstrated that the staining is not highly specific and can also stain necrotic and cell damaged artifacts that are produced during tissue sectioning [14].

Clear evidence of post-ICH apoptosis was provided by Aronowski`s group [15]. For the visualization of post-ICH neuronal apoptosis, these authors used an immune double-staining. NeuN (marker for neurons)-positive neurons were considered apoptotic if stained with cytochrome c. The release of cytochrome *c* has been shown to be a mechanism for predicting apoptosis. Therefore this detection method should be able to provide more precise information. The authors detected numerous cytochrome *c* positive cells in the ipsilateral hemisphere of rats after infusion of autologous blood. The staining was most prominent at 24 h after ICH and then, declined at 3 and completely disappeared at 7 days after ICH. Twenty four hours after ICH, a significant decrease in neurons was observed, indicating that apoptosis significantly contributed to the neuronal death after ICH [15].

2.3. Clinically Relevant Strategies Targeting Mechanisms of Necrosis and Apoptosis after ICH

Preclinical research has led to significant advances in the understanding of mechanisms that contribute to the hemorrhagic stroke cascade leading to cell death and finally resulting in clinical deficits. Despite these findings, treatment still remains the same and is focused on oral anticoagulant reversal and management of blood pressure and other medical complications during the hospital stay. Several potential therapeutic targets showed promising results in animal models, however, were not successful in clinical trials. As described above, programmed cell death is of importance in ICH and understanding these mechanisms and identification of novel therapeutic targets is of paramount interest. This chapter summarizes recent clinical trials and therapeutic targets involving mechanisms of programmed cell death.

2.3.1. Hematoma Removal and Avoidance of Hematoma Expansion (HE)

Intracerebral hemorrhage (ICH) is caused by vessel rupture and subsequent extravasation of blood in the surrounding brain parenchyma. The size of the hematoma is one of the most important predictors of mortality and functional outcome [1]. An association between the size of the initial clot following hematoma expansion (HE) and outcome has

been demonstrated in several studies [16, 17]. Experimental animal studies demonstrated that hematoma removal reduced apoptosis in the surrounding region [18]. Several clinical trials have also investigated the effectiveness of hematoma removal and treatments aimed at minimizing re-bleeding.

Surgical evacuation of the hematoma has been widely investigated in several studies. The large controlled "Surgical Trial in Intracerebral Hemorrhage" (STICH I/II) trials tested early surgery in conscious patients with supratentorial hematomas. The investigators randomized groups into conventional open surgery (craniotomy and hematoma evacuation) and conservative medical management alone and found no beneficial effect for hematoma evacuation. As the subgroup analysis of the STICH I trial revealed a benefit for patients with superficial ICH, the STICH II trial tested patients with superficial hemorrhages. This trial failed to show significant improvement in patients reveiving only medical treatment alone [19, 20]. Given the negative results of these trials, which showed increasing morbidity associated with the surgical approach, studies are now focused on minimally invasive evacuation as well as stereotactic surgery combined with intra-clot thrombolysis. However, to date, no large randomized trial has demonstrated efficacy of these approaches [21-23]. Several groups reported good clinical outcome in patients treated with another technique, decompressive hemicraniectomy (DHC), either with or without hematoma evacuation. However, none of these studies complied with the criteria of a randomized trial and were prone to bias [24-27]. With the need for a large randomized trial, a Swiss multicenter trial is currently enrolling patients in the SWITCH-trial to compare early decompressive craniectomy (DC) with the best medical treatment versus best medical treatment alone [28].

As untreated hypertension is closely linked to ICH and patients with ICH often present with considerably elevated blood pressure [29], blood pressure reduction may limit hematoma expansion. The Intensive Blood Pressure Reduction in Acute Cerebral Hemorrhage Study (INTERACT), INTERACT-2 and Antihypertensive Treatment in Acute Cerebral Hemorrhage (ATACH) trials suggest that aggressive reduction of systolic blood pressure to 140 mm Hg is safe and can reduce hematoma growth [30-32]. As it is important to reduce blood pressure as quickly as possible to avoid early hematoma growth, recent studies have investigating the additional benefits of starting aggressive blood pressure reduction even in the prehospital setting (FAST-BP-trial, NCT01811693) [33]. In summary, recent studies have demonstrated that aggressive reduction of blood pressure may lead to less hematoma expansion with less cellular damage, cellular necrosis, and apoptosis and therefore, may lead to improved neurological outcome.

Besides reducing hematoma growth, hematoma clearance *via* a nonsurgical method may provide another route for protecting cells from programmed cell death. Recently, peroxisome proliferator-activated receptor gamma (PPARγ) agonists attracted attention due to their role in anti-inflammatory and phagocyte-mediated cleanup processes [34]. Moreover, these agonists also diminished apoptosis. PPARγ was shown to bind to NF-κB subunits, directly inactivates NF-κB, and therefore reduce inflammatory responses and secondary brain damage. As stated earlier, CD36 mediated-phagocytosis seems to be another important function of PPARγ-agonists [34-37]. After positive results in animals using rosiglitazone [38], the SHRINC trial (phase 2 study) evaluated pioglitazone in patients with ICH [39]. Results of this study are pending.

2.3.2. Hypothermia

Hypothermia is widely used and recommended as an effective neuroprotective treatment after cardiac resuscitation and has been shown to reduce mortality and improve chances of a good neurological outcome [40]. Brain hypothermia represents a core cooling below the normal body temperature of 37°C, and the largest neuroprotective benefit is estimated to occur at 34°C [41]. Hypothermia has been demonstrated to preserve mitochondrial function, suppress pro-apoptotic pathways and attenuate inflammatory response [42]. This mechanism can not only explain the reduction in brain edema and preservation of the blood-brain-barrier (BBB), but also the improved survival of rats with ICH [43-45]. While a large multicenter trial (EuroHYP trial) has been testing hypothermia in patients with ischemic stroke [46], in patients with ICH only non-randomized trials with small sample sizes have reported a reduced 3-month mortality rate and an attenuation of brain edema development [47, 48]. The results from a phase II randomized trial (CINCH-Cooling in Intracerebral Hemorrhage), in which mildly prolonged hypothermia is being tested, are still pending [49]. However, when considering the side effects of an increased rate of pneumonia and infections [50], a large controlled randomized trial is needed to further assess the potential therapeutic effect of using hypothermia in ICH patients.

2.3.3. Target Oxidative Stress

Oxidative stress is involved in several important stages of pathophysiological responses after ICH. Products of blood degradation such as iron, heme, and thrombin activate the production of free oxygen radicals. These pathological processes increase damage of the cell structures and finally lead to apoptosis [51]. In the various pathways aggravating cell death, several potential therapeutic targets deserve attention.

As mentioned earlier, non-heme free iron is a result of blood clot degradation. Oxidation-reduction reactions of iron ions lead to the generation of free radicals. Therefore preventing this reaction exerts neuroprotection as has been shown in several animal studies [52, 53]. Deferoxamine (DFX) is able to bind iron and may be a therapeutic option in ICH. However, animal studies revealed mixed results. For example, treatment with DFX improved outcome in the whole-blood model, but did not show any effect in the collagenase model [51]. To translate this approach into clinical trials, studies showed that DFX is well tolerated by ICH patients [54]. Currently, the Hi-Def trial [55] (NCT NCT01662895) has been investigating the effects of high-dose deferoxamine in ICH, however, the study was stopped as a result of an incidence of Acute Respiratory Distress Syndrome and modified drug dosages are now under consideration.

Minocycline, another drug involved in several pathways of oxidative stress, showed promising results in several

models of CNS diseases. Minocycline is a semisynthetic tetracycline antibiotic with several neuroprotective and anti-oxidants effects. Besides scavenging free oxygen radicals, it inhibits apoptosis by upregulation of bcl-2 and blocks the activity of matrix metalloproteinases-9 and -12 (MMP-9 and -12, respectively) [56-59]. MMP-9 is detrimental in the acute ICH phase as a result of its role in activation of apoptosis and destruction of basal lamina, but it exerts positive effects in recovery in the subacute phase due to its role in angiogenesis [57, 60, 61]. Therefore, the timing and dosing of minocycline in ICH is essential. Studies in animals showed less edema formation, less inflammation, decreased cell death and attenuated neurological deficits [62, 63]. As no effect on neurological deficits were seen when given at 6 h after collagenase injection, timing is again an important factor and may limit its use in clinical practice [64]. However, minocycline is an attractive therapeutic agent for ICH and is currently being investigated in the Minocycline in Intracerebral Hemorrhagic Patients NCT 01805895 (MACH) trial [33].

Recently, Edaravone, a free radical scavenger, was used to reduce cerebral edema and apoptosis and improved neurological function in an ICH animal model [65, 66]. In ICH clinical trials, the results are not yet clear. A Cochrane analysis of ten Edaravone studies was done in 2011 and concluded that these studies were inconclusive and the quality was poor [67]. A recent study, combining Edaravone with minimally invasive surgery, demonstrated an improvement of National Institutes of Health Stroke Scale (NIHSS) score, however, it did not reduce mortality [68]. Taken together, larger controlled and randomized trials are necessary to evaluate the possible therapeutic effects of Edaravone for treating ICH.

Another free radical scavenger, disufenton sodium (NXY-059), improved post-ICH neurological functions in a rat model [69]. The results of a clinical trial were, however, disappointing. The "Cerebral Hematoma and NXY Treatment trial" (CHANT) showed that, although treatment was safe, no differences in outcome between intervention and control group were detected [70].

Recently, animal studies showed positive results of lipid-lowering medications in ICH. As a result of its antioxidants effects, Cui *et al*. were able to demonstrate that atorvastatin reduced MMP-9 and edema, finally tuned neuronal apoptosis [71], and also improved neurological outcomes [72]. While some studies reported negative effects such as increased thrombogenesis resulting in hematoma growth [73-75], several meta-analyses demonstrated positive results on neurological outcome [76-78]. Thus, large prospective trials are warranted to assess the possible effects of using statin therapy in acute ICH patients.

As stated earlier, matrix-metalloproteinases (MMP) are an important player in response to oxidative stress, but they are also released as a part of the inflammatory response to ICH. Therefore, strategies targeting oxidative stress are closely related to inflammatory cascade.

2.3.4. Target Inflammation

In ICH early activation of microglia, both cell death and decomposition products have been shown to trigger an inflammatory cascade resulting in increased neuronal death. Several studies suggest that attenuation and regulation of immune response may be a promising target in ICH research [79, 80].

FTY720, also known as fingolimod, has been established as an oral treatment for relapsing / remitting multiple sclerosis. The drug was effective in reducing neuronal cell death [81], most likely due to lowering of the ability of inflammatory cells to migrate into the brain after ICH [75]. In animal models of ICH, FTY720 demonstrated efficacy in reducing apoptosis, edema, and brain atrophy [75, 82, 83]. Fu and colleagues tested fingolimod in a human ICH proof-of-concept study. Twenty-three patients with primary supratentorial ICH were tested with either fingolimod or standard management alone. Data showed a significant reduction of edema and neurological deficits in the Fingolimod group [84]. When combining these results, large-scale trials to test the efficacy of fingolimod for reducing brain injury after ICH appear to be promising.

Cyclooxygenase enzymes (COX) are another target of immune system modulation. COX metabolize arachidonic acid to prostaglandins. COX-2 inhibitors exert anti-inflammatory properties, which reduced perihematomal cell death in several animal models [85, 86]. These results lead to a small pilot trial with 44 patients. Indeed, patients treated with celecoxib showed a smaller expansion of perihematomal edema, but it was not powered to reveal an effect on outcome due to the size of the trial with only a small number of patients [87].

Granulocyte Colony-Stimulating Factor (G-CSF), a member of the growth factor family, has been shown to stimulate the proliferation of the neutrophilic granulocyte lineage [88]. However, it is also able to induce anti-apoptotic and neuroprotective effects on neuronal cells, as shown in several animal models [89, 90]. Since studies found a correlation between high serum levels of G-CSF and good outcome after ICH, G-CSF may present another important therapeutic target in ICH [91].

3. LATEST PROGRAMMED CELL DEATH RESEARCH IN ICH

3.1. Pyroptosis

Recent study results indicate that pyroptosis, another kind of programmed cell death, might be significant for ICH-induced brain injury development. Pyroptosis is initiated by nucleotide-binding oligomerization domain-like receptors (NLRs). NLRs recognize pathogen-associated molecular patterns (PAMPs), and their functions are similar to Toll-like receptors (TLRs), another group of receptors able to recognize PAMPs. However, while TLRs are predominantly located in the cell membranes, NLRs recognize PAMPs in the cytosol [92]. Upon binding of the ligand, NLR initiates assembly of a NLR-based multiprotein complex, the inflammasome, which is considered a cellular response to infection or cell damage [93] (Fig. **2**).

To date, the best characterized inflammasome is assembled by NLR pyrin domain containing 3 (NLRP3). It was shown to be involved in the pathogenesis of neurodegenera-

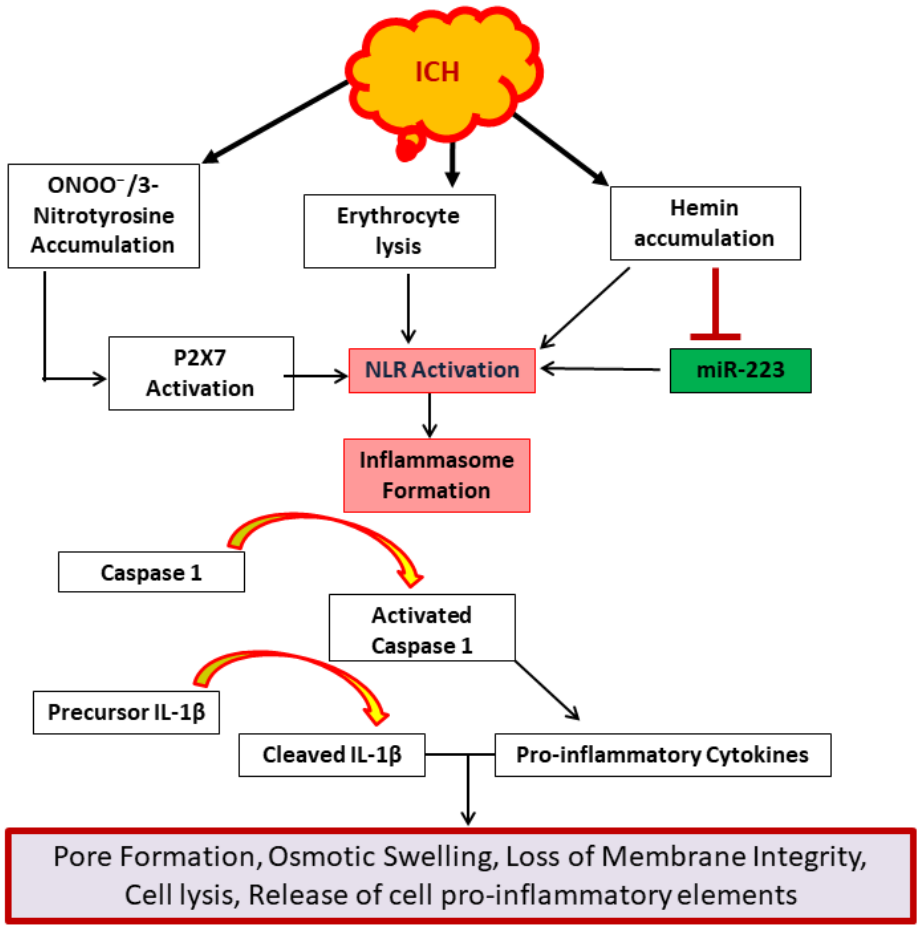

Fig. (2). Schematic presentation of Pyroptosis. Pyroptosis is induced by nucleotide-binding oligomerization domain-like receptors (NLR) located in the cytosol. After binding, NLR initiates assembling a multiprotein-complex, the inflammasome, leading to cell damage.

tive diseases, atherosclerosis, ischemic, and ischemic/reperfusion brain injuries [94-98]. NLRP3 appears to be primarily expressed in microglia and is probably responsible for microglia activation [99, 100]. Inflammasome formation, including the formation of NLRP3, resulted in the recruitment and activation of caspase-1. Caspase-1 activation, in turn, initiated the cleavage and activation of Il-1β and -18. It is mentioning that activation of caspase-1 was not required for apoptosis induction and caspase-1 knock-out did not affect apoptosis and development [101, 102].

Microglia activation, which was shown to increase production of Il-1β and -18, is a component of ICH pathophysiological features leading to the hypothesis that NLRP3-induced pyroptosis may play a significant role in the ICH-induced brain injury development after ICH [103-106]. Unlike apoptosis, pyroptosis does not appear to be immunologically silent and appears to be highly inflammatory. It has been shown to involve pore formation and osmotic swelling of the cell. Pyroptosis has furthermore been characterized by rapid loss of membrane integrity (resulting in cell lysis), the release of proinflammatory elements of the cell, and consequently, in inflammation increase [107]. The increase in caspase-1 appears to depend on inflammation, which consequently leads to cell death; this was demonstrated in ischemic stroke and spinal cord injury models [108-111]. The first indication that caspase-1 induces cell death and participates in development of ICH-induced brain injury was presented by Wo *et al.* [112]. These authors demonstrated that caspase-1 inhibitor could decrease post-ICH brain injury resulting in improved neurological functions and amelioration of brain edema. Furthermore, these authors showed that the inhibition of caspase-1 resulted in the decrease of ICH-induced IL-1β overexpression, reduction of MMP-9 expression and activity, and consequently in decrease in degradation of BBB components. They also indicated that these effects might be mediated by the JNK pathway. Unfortunately, the authors did not directly demonstrate whether ICH could induce expression and/or results in cleavage/activation of caspase-1. They also did not show that the drug produced a decrease in ICH-induced caspase-1activation. Without this information, it can be assumed that the observed beneficial effects of the drug are induced by some side effects. Deriving conclusions regarding the importance of caspase-1 induced cell death on the post-ICH brain injury based on these results is challenging. ICH effects on caspase-1 expression were later investigated by the same group. Using a "blood" ICH mouse model, the authors demonstrated that compared to sham animals (animals received a needle trauma only) hemorrhage caused significant upregulation of caspase-1 production as early as 3 h after ICH [113]. The caspase production peaked 12 h after ICH, however, no significant dif-

ferences between production at 12 and 3 h were observed. The upregulation of caspase-1 production was accompanied by an increase in the production of the NLRP3 inflammasome. In agreement with other studies, the authors demonstrated that NLRP-3 was expressed on microglia, and other brain cell types do not express this inflammasome after ICH. These authors also demonstrated that *in vitro* knock-down of NLRP3 significantly decreased both caspase-1 and IL-1β production, leading to reduced brain edema and improved neurological functions.

Similar results were reported by Feng *et al*. using a rat "collagenase" model. These authors demonstrated that compared to the sham operated animals, ICH induced upregulation of caspase-1 production as early as 6 h after ICH. In this study, the production of NLRP3 increased in a biphasic manner and peaked at 24 and 72 h after ICH [114]. Similar to the results of Ma *et al*. caspase-1 production followed the increase of NLRP3 production. Attenuation of ICH-induced NLRP3 overproduction resulted in a decrease in caspase-1 and IL-1β expressions, indicating that NLRP3 may be a mediator of Il-1β overproduction after ICH. These authors investigated the mechanisms leading to NLRP3 formation and caspase-1 activation. They postulated that ICH-induced activation of P2X7 receptor resulted in the production of peroxonitrite ($ONOO^-$), which in turn was able to initiate the formation of the NLRP3 inflammasome. These authors showed that the temporal profile of P2X7 receptor activation corresponded with the profiles of NLRP3 formation [114]. The profile of $ONOO^-$ formation was not evaluated in this study. However, others demonstrated that the temporal profile of the accumulation of a biomarker for $ONOO^-$ and 3-nitrotyrosine agreed withthe profile of P2X7 and NLRP3 activation [115, 116]. Furthermore, authors demonstrated that both P2X7 receptor and $ONOO^-$ production inhibition significantly attenuated inflammasome activation and IL-1β/IL-18 release, resulting in improved neurological functions [114]. While P2X7 and NLRP3 activation had a single clear peak at 24 h after ICH, caspase-1 activation and 3-nitrotyrosine accumulation were biphasic and, similar to NLRP3 production, reached a maximum at 24 and 72 h after ICH. That is an indication that $ONOO^-$ might indeed be a key player in NLRP3 activation. In contrast, the P2X7 receptor may be only one of the several pathways leading to the development of nitrosative stress and consequent of brain injury after ICH.

Another stimulus, which appears to have induced inflammasome formation was erythrocyte lysis [117]. In a cell culture microglia treated with the erythrocyte lysate for 48 h increased expression of NLRP3. This increase was accompanied by caspase-1 activation [117]. Erythrocyte lysis induced oxidative and nitrosative stresses after ICH; therefore the second, delayed peak of the caspase-1 activation (observed by Feng *et al.*) could be explained by pro-oxidative properties of lysed erythrocytes after ICH, indicating the involvement of oxidative stress in post-ICH NLRP3 formation [114, 118].

In agreement with previous studies, NLRP3 overexpression led to caspase-1 activation and was accompanied by an increase in production of IL-1β and -6 and TNF-α [117]. To investigate the pathway underlying ICH-induced NLRP3 activation, the authors tested the hypothesis that activation of NLRP3 was regulated by microRNA. Using TargetScan, (a system searching for potential targets of microRNA), they detected miR-223 as a potential negative regulator of NLRP3 formation. The authors demonstrated that microglia exposed to lysed erythrocytes expressed less miR-223. Further inhibition of miR-223 resulted in an attenuation of erythrocyte-lysis induced overproduction of pro-inflammatory factors by microglia, thus increasing cell survival rate and decreasing apoptosis. Unfortunately, the authors did not investigate morphological changes induced by miR-233 manipulation. Pyroptosis, unlikely other kinds of programmed death, can be detected morphologically [119, 120]. The investigation of microglia morphological changes caused by miR-233 depression or overexpression would have helped our understanding of present of the presence of pyroptosis in the post-ICH brain. The authors, however, extended their investigation to an *in-vivo* model and, using a "blood" ICH-model, tested whether ICH affects miR-223 expression and whether miR-223 was able to decrease post-ICH-induced brain injury. They demonstrated that in their model, ICH decreased miR-223 expression. An intracerebroventricular injection of miR-223 decreased ICH-induced formation of NLRP3 leading to less brain inflammation, decreased brain edema, and improvement in neurological functions of mice after ICH [117].

The importance of NLRP3 for the increase in inflammation and brain injury after ICH was also confirmed by Weng *et al*. [121], who investigated the role of hemin in NLRP3-upregulation after ICH. Hemin, a break-down product of hemoglobin, is considered a danger-associated molecular pattern (DAMP) [122] and NLRP3 can be activated by DAMP. The participation of hemin in the development of post ICH-induced brain injury has been demonstrated several times [123-125]. However Weng *et al.* investigated for the first time whether the cytotoxic effects of hemin are related to its ability to induce NLRP3 formation. The authors demonstrated that hemin microinjection into the striatum caused a significant inflammatory reaction, which confirmed previous findings with regard to hemin's pro-inflammatory properties [126]. They demonstrated, however, for the first time, that the hemin injection activates NLRP3. Furthermore, those authors determined that hemin effects were mediated by the N-methyl-D-aspartic acid receptor 1 (NMDAR1), which agreed with findings from a previous report [127]. Weng *et al*. showed (that in both mouse brain and microglia cell culture) hemin stimulation resulted in an increase in expression and activation of the NMDAR1 receptor and that NLRP3 activation was dependent on NMDAR1. They further demonstrated that an NMDAR1 inhibitor decreased hemin-induced activation of microglia lead to attenuation of Il-1β microglia production. Unfortunately, in this publication no cellular morphological evaluation was done. Although there is a clear indication of presence of pyroptosis after ICH, further investigation needs to be done.

3.2. Autophagy

Autophagy is an intracellular system responsible for the degradation and recycling of most cellular components. During this process, cells sequester and degrade parts of their

own cytoplasm, including organelles. The degradation of cellular proteins and the turnover of dysfunctional organelles occurs within a unique type degradative organelle called an autophagic vacuole [128, 129].

There are three different forms of autophagy: 1) macroautophagy; 2) microautophagy; and 3) chaperone-mediated autophagy [130]; these processes are involved in numerous physiological processes and their functions vary depending on the tissue. This form of cell death is especially important for cells (such as neurons) that do not divide after differentiation. Autophagy can recycle damaged organelles, thus contributing to maintenance of cellular integrity. For instance, damaged mitochondria can be removed by autophagy. This process leads to prevention of uncontrolled release of ROS and protection of cells from oxidative stress.

Several signaling pathways induce autophagy *via* several mechanisms: 1) oxidative stress; 2) inflammation; 3) accumulation free iron; these are important factors of post-ICH pathophysiology, and all appear to be able to induce autophagy.

Autophagy is considered as a caspase-independent cell death pathway. It has been demonstrated that another death-inducing signaling molecule, cathepsin D, can initiate authophagy [130, 131]. This biomarker (cathepsin D) for autophagy was used by He *et al*. in their study reporting the presents of autophagy after ICH [132]. Using a "blood" ICH model the authors demonstrated that there was a significant increase in the cathepsin D production in the ipsilateral *versus* contralateral hemisphere as early as one day after blood injection. Cathepsin D production was evaluated by western blot. It peaked at day 7 and remained upregulated until day 28. The potential presence of autophagic cell death was confirmed by western blot using another marker, microtubule-associated protein light chain 3 (LC3). LC3 is localized to the autophagosomes, a key structure in macroautophagy. It exists in two forms, LC3-I and -II. While LC3-I is a cytosolic protein, LC3-II is membrane-bound and a significant increase in LC3-II concentration has been observed during autophagosome formation [133]. At day 7, He *et al*. observed a significant increase in the LC3-I/LC3-II ratio in ipsilateral hemisphere of ICH animals compared to the contralateral hemisphere of ICH animals and also to the ipsilateral hemisphere of sham animals. Furthermore, immunohistochemical and morphological analyses of cells, expressing this autophagy marker revealed that one day after ICH, most cells expressing cathepsin D in the perihematomal area were neurons. However, at days 3 and 7 only a few cathepsin D positive cells were neurons and the marker was mostly expressed in glia-like cells. Additionally, the authors monitored the formation of autophagic vacuoles using the electronic microscopy, which is the most accurate and sensitive method of autophagy monitoring [134]. They found numerous cells with vacuoles containing membranous structures and parts of the cytoplasm which were most prominent at day 7. The time point of vacuoles formation agreed with time point of maximal cathepsin D expression. The results of this electron microscopy study also agreed with immunohistochemical/morphological analyses and revealed that most of the autophagic vacuoles containing cells were glia cells. To understand which pathophysiological factor induced autophagy, the authors injected ferrous iron into the brain of animals. They detected a significant increase of cathepsin D in the ipsilateral hemisphere of iron injected animals 24 h after injection. Most of cells appeared to be neurons. Interestingly, others demonstrated that although significant accumulation of iron after blood injection could not be observed until day 3, the first iron positive cells were observed as early as one day after ICH and these iron-positive cells were neurons [135]. Iron positive glia were detected at day 3 [135]. At day 3, He *et al*. showed the presence of autophagy in the glia cells [132]. This agreement in time points indicated that iron might be indeed the factor inducing autophagy after ICH. Furthermore, He *et al*. demonstrated that an iron chelator, deferoxamine, significantly decreased expressions of autophagy markers. Unfortunately, the authors did not evaluate neurological functions of animals. The effects of deferoxamine on this parameter are controversional. While some groups demonstrated improvement of neurological functions after post-ICH deferoxamine administration [136], another group showed that deferoxamine administration did not result in the significant improvement of neurological functions [137]. It is, therefore, hard to say if autophagy serves as a protective mechanism or autophagy, due to dying some certain neurons neurons population, is the normal course of neurological dysfunctions after ICH.

The same working group further investigated the role of thrombin in induction of post-ICH autophagy [138]. Hu *et al*. infused 3U of thrombin into the brain of animals and examined whether the infusion would change expressions of autophagy markers. The thrombin infusion increased production of cathepsin D as early as one day after thrombin infusion. The production remained upregulated for three days and then returned to the normal level at day 7. This effect was accompanied by conversion of LC3-I to LC3-II at days 1 and 3. The authors also showed the presence of autophagosomes in the brain of thrombin animals three days after injection, and they also studied the thrombin in the cultured astrocytes. Similar to an *in vivo* study, thrombin induced LC3-I/LC3-II conversion in an astrocyte cell culture. The effect was dose-dependent. Interesting enough, 3U of thrombin (the thrombin activity was used *in vivo* part of this study) was unable to induce statistically significant effects and only an increase of thrombin activity to 5U triggered the LC3-I to LC3-II conversion, indicating that sensitivity of brain cell to thrombin might vary from type to type. The effect was also time-dependent. While no effect of thrombin was observed 1 h after treatment, a significant effect of 5U thrombin was observed 6 h after treatment. The autophagy marker was upregulated up to 24 h and returned to normal 48 h after treatment.

Most importantly, the authors investigated whether thrombin-induced formation of autophagic vacuoles was protective or associated with increased astrocytic death. In order to test this hypothesis, the authors used 3-Methyladenine (3-MA), an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), which is intricately involved in autophagy formation [139-141]. The authors found that autophagy was protective. 3-MA decreased the formation of autophagic vacuoles, lead-

ing to aggravation of thrombin and an increase in cell death [138].

The effect of autophagy inhibition on the development of secondary brain injury after ICH was also investigated by others. Yang *et al*. hypothesized that activation of toll-like receptor 4 (TLR-4) triggered the microglial autophagy and consequently microglial activation, thus triggering the development of post-ICH brain injury [142]. To test their hypothesis they injected autologous blood into the brain of wild-type or TLR-4 knockout mice and observed a significant increase in brain water content and neurological dysfunction in both wild-type and KO animals. Intracerebralventricularly administered 3-MA attenuated the ICH-induced increase in brain water content and improved neurological functions of wild-type animals, again indicating the protective features of autophagy. The authors confirmed these results in an *in vitro* study. They demonstrated that incubation with erythrocyte lysate or LPS activated microglia, and microglia activation was then accompanied by formation of autophagosomes. Inhibition of autophagy with 3-MA not only decreased the number of autophagosomes but also attenuated pro-inflammatory cytokine release and the ability of microglia to migrate.

These results agreed with another study, in which autophagy inhibition induced cell death in an *in-vitro* model of ICH was demonstrated [138]. The results disagreed, however, with the *in-vivo* part of the same study, demonstrating that anti-thrombin therapy decreased post-ICH autophagy [138]. It is well known that anti-thrombin therapy after hemorrhagic stroke results in less brain damage [143, 144]. Hence, the autophagy inhibition could be beneficial. It is possible that the protective effects of thrombin are mediated by other, autophagic-independent pathways [143, 144].

Yuan *et al*. confirmed the role of post-ICH autophagy on post-ICH microglia activation [145]. Authors used an in-vitro ICH model and treated the microglia culture with erythrocyte lysate. Incubation with erythrocyte lysate increased the time-dependent production of another autophagic marker, MAP1LC3B. The increase was associated with an increase in production of pro-inflammatory cytokines, and inhibitors of autophagy decreased erythrocyte lysis induced microglia activation. In contrast, treatment with autophagy activators resulted in aggregation of microglia activation. The authors also investigated the possible molecular pathways underlying autophagy activation by silencing autophagic proteins. The siRNA mediated knock-down of such canonical autophagic proteins as Beclin1 and ATG5 decreased erythrocyte lysis induced microglia inflammation, indicating that the classical Beclin-1/ATG5 pathway is involved in microglia activation. The authors also investigated whether or not activated microglia damaged neurons. In close agreement with previous publications, activated microglia induced injury to neuronal cells. Autophagic inhibition in the microglia resulted in a significant decrease in neuronal death. Furthermore, authors confirmed findings of *in vitro* study by testing effects of autophagy inhibitors or activators in an in-vivo ICH model. Authors injected 20-µl of autologous blood in the brain of mice and demonstrated that while the administration of autophagy inhibitors reduced the ICH-induced increase in brain water content and improved neurological functions, autophagy activators aggravated both development of post-ICH brain edema and neurological dysfunctions. Unfortunately, a time-dependent investigation of autophagy marker expression was not carried out in this study, and the method and time point of drug administration (intracerebral ventricular injection, 10 min after ICH) was not really clinical relevant. It is therefore difficult to comprehend whether the results are applicable to clinical situations. Understanding whether anti-autophagy therapy might provide a novel strategy for ICH treatment requires further experiments.

Similar information was provided by Riu *et al*. who, using an autologous blood mouse model, investigated the time-dependent profile of post-ICH Bnip3L expression [146]. Bnip3L-functions are not completely understood. There are, however, indications that Bnip3L induced delayed neuronal death after a stroke that triggered excessive mitophagy. Since mitophagy is a process of selective mitochondrial degradation by autophagy [147], Bnip3L appears to be a useful marker. Authors observed the statistically significant increase in Bnip3L expression as early as 6 h after ICH induction. Bnip3L remained upregulated 24 h after ICH, slightly declined at 48 h and returned to normal levels 72 h after ICH. Authors also demonstrated that Bnip3L was mostly expressed in cytoplasm of neurons and that the Bnip3L expression profile temporarily matches the development of neurological dysfunctions. Furthermore, the authors mentioned that Bnip3L might induce apoptosis *via* the caspase-3 pathway. In order to test the hypothesis that Bnip3L is involved in neuronal apoptosis after ICH, the authors conducted an immunohistochemical study and showed co-localization of Bnip3L/active caspase-3 in addition to activation of caspase-3 in neurons three days after ICH. As a result of these findings, the authors concluded that Bnip3L might be involved in neuronal apoptosis. It is, however, worthwhile mentioning that autophagy and apoptosis often occur in the same cell and autophagy mostly precedes apoptosis [148]. Therefore one can not completely exclude the presence of autophagy in these cells. Besides Riu *et al*. showed active caspase-3 colocalizated with Bnip3L three days after ICH. At this time point, the Bnip3L expression went back to the basal level, however, the basal expression of the protein was relatively high. Due to the high level of basal expression, it was not surprising that most caspase 3 positive cells were also Bnip3L positive. To further evaluate the association between Bnip3L and neuronal apoptosis after ICH, additional experiments need to be done.

Furthermore the match between the increase of the Bnip3L and development of neurological dysfunctions might be a coincident observation. Bnip3L expression upregulation was observed as early as 6 h after ICH, and significant neurological deficits were present at this time point. It is somehow difficult to believe early neurological deficits (6 h after ICH) were caused by neuronal death. The deficits might be consequences of mechanical brain damage induced by needle and postoperative trauma. The relation between apoptosis and Bnip3L in neuronal death after ICH has not been completely established. Nonetheless, results clearly indicate, that

autophagy might play a significant role in the development of secondary ICH-induced brain injury, and manipulation of autophagy may represent a novel therapeutic strategy for post-ICH patients.

CONCLUSION AND FUTURE DIRECTIONS

ICH is still associated with high mortality and morbidity and the discrepancy between clinical and pre-clinical studies is disappointing. However, the understanding of new mechanisms and the developing of several drugs, which target well established pathological pathways leading to cell death after ICH, bring new hope for ICH patients. Modulation of immunological response after ICH showed promising results in a small proof-of-concept study and needs to be verified in a larger trial. So far, none of existing therapeutic approaches target newly described types of programmed cell death: autophagy and pyroptosis. Improved understanding of ICH-induced pathology and underlying mechanisms will help in development of new therapeutic approaches, adjustment of the time frame application of existing treatment, and improve a successful transition from bench to bedside.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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