Glycyrrhizic acid confers neuroprotection after subarachnoid hemorrhage via inhibition of high mobility group box-1 protein: A hypothesis for novel therapy of subarachnoid hemorrhage

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A B S T R A C T

Subarachnoid hemorrhage usually results in poor clinical outcome and devastating neurological deficits. The early brain injury and delayed vasospasm after subarachnoid hemorrhage (SAH) are involved in the poor prognosis to the patients, while the mechanisms have not been well elucidated. Previous studies found an up-regulation of Toll-like receptor 4 (TLR4), inflammatory factors and high-mobility group box 1 (HMGB1) in the cortex after SAH. Increased inflammatory response contributes to the early brain injury and delayed vasospasm after SAH. Moreover, we found that the inflammatory response could be induced and amplified following recombinant HMGB1 (rHMGB1) addition in cultured neurons. Based on the latest researches in this field, we raised a hypothesis that HMGB1, a prototypical member of damage-associated molecular pattern (DAMP) family, could be passively released from the damaged neuroglia cells and hemocyte lysis after SAH. Extracellular HMGB1 initiated the inflammation through its receptors. The inflammatory mediators then acted on the neurocytes to make them actively release HMGB1 continuously, manifesting an double phases. HMGB1 might be the key factor to induce sterile inflammation, and thus be one of the origin of early brain injury and delayed vasospasm after SAH. Inhibition of extracellular HMGB1 activities might be a novel therapeutic target for SAH to reduce the damaging inflammatory response. Glycyrrhizic acid (GA) which was extracted from liquorice and confirmed as a nature inhibitor of HMGB1 with little side-effects could inhibit extracellular HMGB1 cytokine activities and reduce the level of inflammatory response, thus alleviating early brain injury and cerebrovasospasm. GA might be a new novel therapy of SAH for better outcomes.

Introduction

Subarachnoid hemorrhage (SAH) is a life-threatening cerebral vascular disease. It contributes to 7% of the stroke [1]. Despite of its relatively low incidence, its early age of onset and poor outcome result in a lot of life years lost. Recent studies have indicated that early brain injury and delayed vasospasm contributed to the poor outcome of SAH patients [2,3]. More and more evidence has shown that inflammation triggered by SAH is involved in both processes [4–6]. Up-regulated expression of toll-like receptor4 (TLR4), inflammatory factors were detected both in cortex tissue and subarachnoid arteries after SAH [7–9]. Inflammation triggered by SAH enhances the breakage of blood brain barrier, brain edema, cell apoptosis and death, which in turn aggravate inflammatory response [8,10,11]. Although the detailed mechanisms about the relationship between inflammation and cerebrovasospasm are not totally understood, the result that inhibition of the inflammation process could relieve delayed cerebrovasospasm which was proved in SAH models confirms their relationship [3,12,13].

High-mobility group box 1 (HMGB1), as a nonhistone protein binding with DNA, exists in the nucleus of nearly all eukaryotic cells and facilitates gene transcription under physiological conditions [14]. In pathological state, HMGB1 could be passively released from the damaged neuroglia cells and hemocyte lysis after SAH [8,10,11] (as shown in the right part of Fig. 1) [14–15]. Extracellular HMGB1 serves as damage-associate molecular pattern (DAMP) and mediates inflammatory response after interaction with toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4) and receptors for advanced glycation end-products.

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(RAGE) [14,16,17] (as shown in the left part of Fig. 1). Both experimental and clinical studies have shown that the level of HMGB1 was significantly up-regulated and remained a high level during the process after SAH [18,19]. Clinical researches also indicated that HMGB1 level correlated significantly with the levels of inflammatory factors (including IL-6, IL-8, and TNF-α) in the cerebrospinal fluid (CSF) [19]. Besides, HMGB1 level was reported as an independent predictor of poor functional outcome and mortality after 1 year, in-hospital mortality and delayed cerebrovasospasm [19,20]. Several studies in brain ischemia, spinal cord injury, and traumatic brain injury models have shown that treatments targeting at inhibition of HMGB1 had beneficial effects through inhibiting inflammatory response [21–23]. Moreover, in our previous study, we found that the inflammatory response could be induced and amplified following recombinant HMGB1 (rHMGB-1) addition in cultured neurons [24].

Above all, the evidence indicated the key role of HMGB1 in early brain injury and delayed cerebrovasospasm after SAH.

Glycyrrhizic acid (GA), a nature anti-inflammatory drug was found to be a novel pharmacological inhibitor of extracellular HMGB1 cytokine activities [25,26]. Recent studies show that GA binds directly to HMGB1 and identify its binding surface on HMGB1 [27]. GA might present neuroprotection through its inhibition of HMGB1 cytokine activities [25]. Treatments targeting at HMGB1 after SAH might protect neuroglia cells from death or apoptosis, alleviate early brain injury and cerebrovasospasm, thus improving outcomes. This article is to state the hypothesis in detail.

Hypothesis

Based on the above evidence, we propose the hypothesis that HMGB1, a prototypical member of damage-associated molecular pattern (DAMP) family, could be passively released from the damaged neuroglia cells and hemotocyte lysis after SAH. Extracellular HMGB1 initiated the inflammation through its receptors (including TLRs and RAGE) (as shown in the left part of Fig. 1). The inflammatory mediators then acted on the neurocytes and immune cells to make them actively release HMGB1 continuously, manifesting an double phase (as shown in the right part of Fig. 1). Up-regulated HMGB1 and inflammation in brain tissue and artery walls could be involved in the pathological processes of early brain injury and the delayed cerebrovasospasm. HMGB1 might be the key factor to induce sterile inflammation. Thus inhibition of extracellular HMGB1 cytokine activities may be a novel therapeutic target for SAH to reduce the damaging inflammatory response, thus alleviating the following early brain injury and the delayed cerebrovasospasm. GA was reported to be a nature inhibitor of HMGB1. Therefore, GA might present neuroprotection through its inhibition of HMGB1 cytokine activities.

The hypothesis was quite different from previous studies in inflammatory signal pathways after SAH. This hypothesis intended to explore the origin of the inflammatory response after SAH. Previous studies in SAH have revealed that inflammatory response played a pivotal role in the process of SAH, both in early brain injury and cerebrovasospasm [4,5,28,29]. However, it is still not totally understood where the inflammation came from or what kind of molecules triggered the inflammatory response. According to the latest researches, inflammation was initiated by two kinds of models, pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) [30,31]. Inflammatory response after SAH was widely recognized as a sterile inflammation which was usually caused by DAMP [31]. We propose the hypothesis to find the DAMP which caused the inflammatory response after SAH and use the effective inhibitor to cut the source of the damaging inflammatory response and improve the poor outcome of SAH.

Fig. 1. Representative Picture showed the mechanism of our hypothesis. Increased extracellular HMGB1 after SAH triggers inflammation and increases downstream inflammatory factors through TLRs/NF-κB and RAGE/NF-κB signal pathway (as shown in the left part of Fig. 1) [16]. Up-regulated inflammatory factors enhance the expression of HMGB1 and help HMGB1 relocation from nucleus to outside the cells (as shown in the right part of Fig. 1) [14]. HMGB1 is in the center of the feed-forward regulation of the damaging inflammatory response and might be the source of damaging inflammatory response after SAH.
Recent researches played wide attention to the research of HMGB1 which was a prototypical member of DAMP family [14,17,30,31]. Extracellular HMGB1 was recognized as a danger signal which could initiate similar inflammatory response as PAMP. Current studies have shown that increased HMGB1 level could be observed in brain tissue of SAH animal models and in CSF of SAH patients [18,19]. We suppose that passive release from damaged cells and active secretion from immune cells contributed to the increased level of HMGB1 after SAH. Part of them came from breakdown of blood cells trapped in subarachnoid space which occurs by 16–32 h after SAH [4,32]. Moreover, SAH caused neuroglia cells apoptosis and death as early as 10 min after SAH [33]. HMGB1 level in CSF ascended significantly after the large passive release of HMGB1 from hemolytic nucleated blood cells and cortical damaged neuroglia cells, the latter could also be observed in cerebral ischemia models [17]. Extracellular HMGB1 released rapidly from these damaged cells mediated cross-talk between injured cells and relatively healthy cells around damaged tissues. Combination of HMGB1 with its receptors, including TLR2, TLR4 and RAGE, induced the TLRs/NF-kB, RAGE signal pathway and promoted the production of pro-inflammatory factors, including IL-1β, TNF-α and up-regulated metalloproteinase-9 (MMP9) [16,34] (as shown in the left part of Fig. 1). When exposed to the high pro-inflammatory factors, relatively healthy neurocytes around damaged tissues and immune cells in local or migrated by attraction of high pro-inflammatory factors secreted the HMGB1 actively with up-regulated HMGB1 mRNA level, which in turn promoted more violent inflammatory response via its receptors [14] (as shown in the right part of Fig. 1). Elevated inflammation contributes to breakage of blood brain barrier, brain edema, cell apoptosis and death, which aggravate inflammatory response again and enhance the poor outcome [8,10,11].

Accumulating evidence demonstrates that inflammation has a strong relationship with delayed cerebrovasospasm [4,12]. The pathological alterations related to inflammation, such as the leukocyte recruitment, infiltration and activation, have been observed in both experimental and clinical studies of cerebrovasospasm [35,36]. Many molecules involved in inflammatory response, including cytokines, adhesion molecules, complement, and immunoglobulin, were also detected to change in the cerebrospinal fluid (CSF), plasma or arterial wall in patients or experimental models of cerebral vasospasm [37–39]. Moreover, inhibition of inflammation or NF-kB activities could alleviate delayed cerebrovasospasm [3,40]. Thus we hypothesized that HMGB1 as an origin of inflammation and NF-kB activities (as shown in the left part of Fig. 1), could be responsible for the delayed cerebrovasospasm. Up-regulated HMGB1 and inflammation in endothelial cells and artery walls could be involved in the pathological processes of the delayed cerebrovasospasm.

As is described above, HMGB1 was located in the center of the vicious circle of damaging inflammatory response signal pathway and brain injury pathological process, which suggested that treatment targeting at HMGB1 might reduce the damaging inflammatory response after SAH and relieve early brain injury and delayed cerebrovasospasm after SAH. GA is a novel pharmacological inhibitor of HMGB1 cytokine activities [25] and confers anti-inflammatory effect in the clinical treatment of hepatitis [26]. Glycyrrhizic acid (GA) bound directly to HMGB1 and identified its binding surface on HMGB1 (helix 1 and 2 in the HMG boxes), interfering only mildly with the binding of HMGB1 to DNA in living cells [27]. NMR and fluorescence studies indicate that GA could inhibit extracellular HMGB1 cytokine activity through blocking its binding site [25]. These features helped GA inhibit extracellular HMGB1 activities with little influence on the physiological effect of HMGB1. Researches in ischemia brain injury and ischemic spine core injury models suggested that using GA could inhibit HMGB1 activities and lower downstream inflammatory factors, thus alleviating the injury [22,23]. These results indicate that GA might have an insight in its neuroprotection effects through its inhibition of HMGB1 activities after SAH.

Evaluation of the hypothesis

The primary injury caused by increased intracranial pressure and decreased intracranial perfusion pressure could not been easily reversed, but the following neurocytes injury caused by damaging inflammatory response and delayed cerebrovasospasm could be alleviated to improve outcome if treated adequately [5]. HMGB1 was widely recognized as a member of DAMP family, which was studied in detail in brain ischemia models [23,41]. However, little study about the role of HMGB1 could be found in SAH researches. This hypothesis was the first to propose that extracellular HMGB1 was one of the origins of the damaging inflammatory response and brain injury pathological process after SAH and suggest a possibility to use GA as a novel drug through its effect on the HMGB1 to improve outcome of SAH patients.

Testing the hypothesis

To test the hypothesis, three main steps are suggested to take. The first step is to identify the pro-inflammatory role of HMGB1 after SAH. Although our previous study found that the inflammatory response after SAH could be induced and amplified following recombinant HMGB1 (rHMGB1) addition in cultured neurons, in vivo study is still required to understand whether HMGB1 could initiate the inflammatory response [24]. Inflammatory factors which could reflex inflammatory response in cortex and arteries after rHMGB1 addition to the subarachnoid space will be tested.

The second step is to identify the effect of GA in early brain injury after experimental SAH models. The animals will be randomly divided into five groups: saline control group, SAH group, SAH + vehicles, SAH + GA (5 mg/kg), SAH + GA (10 mg/kg). GA will be injected from vein 1 h after SAH. According to previous studies, the highest mortality was observed in the first 24 h after SAH [42], thus the animals will be arranged to be sacrificed and detected 24 h after SAH. HMGB1 protein and mRNA levels would be examined by western blot and real-time PCR. Inflammatory factors, such as IL-1β, TNF-α would be measured by ELISA and real time PCR. FJC and TUNEL will be used to identify the cell death and apoptosis after SAH.

The third step is to detect the effect of GA in delayed cerebrovasospasm after SAH. The animals will be randomly divided into five groups: saline control group, SAH group, SAH + vehicles, SAH + GA (5 mg/kg), SAH + GA (10 mg/kg). GA will be injected from vein once per day after SAH. Delayed cerebrovasospasm was observed in day 3 and day 5 after SAH, thus the animals will be sacrificed in day 5 after SAH. HMGB1 protein and mRNA levels in artery tissue would be detected by western blot and real-time PCR. Inflammatory factors, including IL-1β, TNF-α would be detected by ELISA and real-time PCR. HE staining and diameter of and cerebral arteries (middle cerebral artery and basal artery) will be employed to evaluate the cerebrovasospasm and the treatment effect of GA.

The above three animal experiments will be tested in at least two kinds of different SAH models to confirm the role of GA after SAH. In detail, prechiasmatic injection SAH model and suture models will be employed to study role of GA in the early brain injury while double hemorrhage models and prechiasmatic injection model will be used to check the role of GA in the study of delayed cerebrovasospasm.

The fourth step is the clinical researches. After the above three steps were completed and GA was proved to be neuroprotection in

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animal SAH models, the fourth step could be considered to carry out. Some healthy volunteers and SAH patients in same Hunt–Hess grade will be selected in the research. These patients were be divided into healthy volunteer group, SAH group, SAH + placebo, SAH + GA, the inflammatory factors in CSF in patients of different groups will be tested and cerebral angiography will be employed to detected the cerebrovasospasm.

Consequences of the hypothesis and discussion

The pathological process about the brain injury after SAH is not completely understood. Increased intracranial pressure, decreased cerebral perfusion, and decreased cerebral blood flow (CBF) contribute to the primary injury after SAH which were difficult to reverse [5]. Thus studies in SAH mainly focused on the secondary injury caused by damaging inflammatory response, oxidative stress, and delayed cerebrovasospasm after SAH [5,13]. If our hypothesis did work out, that means HMGB1 is one of the important origins of the damaging inflammation. The result will help us understand the pathological process after SAH and direct us to find effective drugs to inhibit the harmful process. Previously, researchers employed drugs to inhibit the key adapters of the signal pathways to inhibit the damaging inflammatory pathway, which revealed improved outcome in animal models [11]. But there were risk that inflammatory response to microorganism invasion could also be reduced. Directly inhibition of extracellular activities of HMGB1 would be the novel therapy for reducing damaging inflammatory response. Several drugs were reported to have the ability to inhibit HMGB1 release from the nucleus into the extracellular space, without affecting its mRNA or protein levels, aging inflammatory response. Several drugs were reported to have activities of HMGB1 would be the novel therapy for reducing dam-


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