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Bex1 attenuates neuronal apoptosis in rat intracerebral hemorrhage model

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ABSTRACT

Brain expressed x-linked gene 1 (Bex1) which is at high levels in several populations of central nervous system (CNS) neurons, belongs to a family of small proteins of unknown function, playing roles as adaptors or modulators of intracellular signaling pathways. But its distribution and function in CNS remains unclear. Neuronal apoptosis is the major pathogenesis in secondary brain injury of intracerebral hemorrhage (ICH). In this study, the roles of Bex1 were explored in the pathophysiology of ICH. Western blot, immunohistochemistry, and immunofluorescence showed that obvious up-regulation of Bex1 in neurons adjacent to the hematoma after ICH. Furthermore, the increase of Bex1 expression was accompanied by the enhanced expression of Bax and active caspase-3, and decreased expression of B-cell lymphoma 2 (Bcl-2) following ICH. The in vitro study using Bex1 siRNA transfection in hemin-exposed PC12 cells suggested that Bex1 exerted anti-apoptotic function. Therefore, Bex1 may play the neuronal anti-apoptosis role following ICH, implying a novel molecular target for the therapy of ICH.

1. Introduction

Intracerebral hemorrhage (ICH) is defined as bleeding into the brain parenchyma, which accounts for 10–15% of total strokes and the percentage is even larger in Asians [1]. Although ICH patients are with a high rate of morbidity and mortality, as well as there are numerous of clinical and basic researches, there still lacks effective medical and surgical strategies [2]. Therefore, there is an urgent need to elucidate the cellular and molecular mechanisms of ICH.

Rupture of blood vessels within brain parenchyma leads to the primary and secondary injuries. Hematoma formation, brain edema, and the activation of cytotoxic, oxidative and inflammatory pathways all induce the brain injury after ICH [3]. These parallel pathological pathways lead to neuronal death and subsequent neurological impairments. Neuronal apoptosis is considered as one of the most crucial events including extrinsic and intrinsic cell death pathways [4]. The extrinsic pathway requires cell surface receptors binding to their specific ligands and then activating caspase-8 [5], while the intrinsic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) events, in which B-cell lymphoma 2 (Bcl-2) family members play vital roles. Bax, a member of Bcl-2 family, promotes apoptosis by translocating from the cytosol to the mitochondrial outer membrane (MOM) [6]. On the contrary, Bcl-2 is an inhibitor of the mitochondrial apoptotic pathway through preventing the release of cytochrome c and the activation of caspase [7]. Additionally, caspase-3 is a key mediator of neuronal apoptosis and can be activated by both extrinsic and intrinsic pathways [8].

Brain expressed x-linked gene 1 (Bex1) belongs to a small growing family, which includes six members having high homology of gene sequences and structures with different expression patterns and subcellular localization [9]. Bex1 acts as a candidate tumor suppressor gene for its inactivation promotes the development of multiple tumors such as malignant glioma, oral squamous cell carcinoma, pediatric intracranial ependymoma [10–12]. In addition, Bex1 plays key roles in the formation of multiple signaling network hubs [13]. Particularly, Bex1 is a regulator of neuron regeneration, as Bex1 knockout mice are

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deficient in axon regeneration after sciatic nerve injury [14]. Interestingly, Bex1 protein level is cell-cycle dependent in PC12 neuronal cells, with the lowest expression level in G1 phase and the highest level in S phase. Moreover, down-regulation of Bex1 is necessary for the cell cycle exit of neural progenitor cells, as overexpression of Bex1 results in sustained proliferation even under growth arresting conditions [15]. Previous studies suggest a role for BEX1 in skeletal muscle differentiation, as well as a role as in downstream nerve growth factor (NGF) signaling [16,17]. Recent research also reveals that BEX1 functions as an mRNA-dependent effector that augments pathology-promoting gene expression such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) during heart failure [18]. However, the expression and function of Bex1 in ICH are unclear.

In this study, the expression and function of Bex1 in ICH are detected in vivo and in vitro, suggesting that Bex1 attenuates neuronal apoptosis after ICH, which is the potential target for the pharmacotherapy of ICH.

2. Methods and materials

2.1. Rat ICH model

Male Sprague-Dawley rats (230–275 g) were provided by the Experimental Animal Center, Nantong University (Nantong, Jiangsu, China) and were kept at 21 °C on a 12 h light-dark cycle. The rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) positioned in a stereotaxic frame, and a cranial burr hole (1 mm in diameter) was drilled near the right coronal suture 3.5 mm lateral to the midline. Autologous whole blood (50 μl) was collected from its tail tip to a sterile syringe. The sterile syringe was inserted stereotactically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma), with the rate of 10 μl/min [19]. After 10 min, the needle was removed, the skin incision was closed, and the animals were allowed to recover. Sham rats only had a needle insertion. Experimental rats (n = 5 per time point) were sacrificed at 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d and 14 d, respectively. The sham rats were sacrificed on 3 d. Animal experiments were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory and approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch. All efforts were made to minimize the number of animals used and their suffering.

2.2. Forelimb placing test

The rats were held by torsos, thus their forelimbs could hang free. Independent testing of each forelimb was elicited by brushing the respective vibrissae on the corner edge of a countertop. Intact rats lay up the forelimb quickly onto the countertop. According to the extent of injury, placing of the forelimb contralateral to the injury may be impaired. During the experiments, each rat was tested ten times for each forelimb and the percentage of trials in which the rat placed the left forelimb was calculated [20].

2.3. Corner turn test

The rats were allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the rat should turn either to the left or to the right. Only the turns involving full rearing along either wall was included (a total of eight per animal). According to the extent of injury, rats may show a tendency to turn to the side of the injury. The percentage of right turns was used as the corner turn score. The rats were not picked up instantly after each turn so that they would not develop an aversion for their prepotent turning response [21].

2.4. Protein extraction and western blot analysis

After injected an overdose of sodium pentobarbital (50 mg/kg), rats were executed at different time points postoperatively, and the brain tissue around the hematoma (extending 2 mm to the incision) as well as an equal part of the normal, sham, and contralateral cortex were collected and stored −80 °C until use. To prepare the lysates, frozen samples were weighed and minced on ice. The samples were then homogenized in lysis buffer (1% NP-40, 50 mmol/l Tris, pH = 7.5, 5 mmol/l EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) and centrifuged at 12,000 rpm, 4 °C for 20 min to collect the supernatant. After ascertain of its protein concentration with the Brad-ford assay (Bio-Rad, Richmond, CA, USA), protein samples were separated by SDS-PAGE and transferred to a PVDF membrane by a transfer apparatus at 250 mA for 2 h. The membranes were blocked with 5% non-fat milk for 2 h and incubated with primary antibodies against Bex1 (mouse, 1:1000; Abcam, Cambridge, MA, USA), active caspase-3 (rabbit, 1:1000, Cell Signaling Technology, Beverly, MA, USA), GAPDH (rabbit, 1:2000, Abcam), Bcl-2 (mouse, 1:1000, Abcam), and Bax (rabbit, 1:1000, Cell Signaling Technology) at 4 °C overnight. Finally, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h and visualized using an enhanced chemiluminescence system (ECL; Invitrogen, Carlsbad, CA, USA).

2.5. Immunohistochemistry

Rats were deeply anesthetized and perfused with saline and then 4% paraformaldehyde through the ascending aorta at the appointed time points. After perfusion, the brains were collected and post-fixed in the same fixative for 3 h and then replaced with 20% sucrose for 2-3 days, followed by 30% sucrose for another 2-3 days. Tissues were then cut at 7 μm with a cryostat and stored at −20 °C until use. Slide-mounted sections were kept in an oven at 37 °C for 30 min, and rinsed twice in 0.01 M PBS for 5 min. Then the sections were handled with 10 mmol/l citrate buffer (pH = 6.0) and heated to 121 °C in an autoclave for 3 min to retrieve the antigen. The sections were taken from the pressure cooker and cooled to room temperature (RT) spontaneously. Then, the sections were blocked with confining liquid including 10% donkey serum, 1% BSA, 0.3% Triton X-100 and 0.15% Tween-20 for 2 h at RT, then incubated with anti-Bex1 antibody (mouse, 1:300, Abcam) overnight at 4 °C. Following incubation in the secondary antibody at 37 °C, the sections were color-reacted with 0.02% DAB, 0.1% PBS, and 3% H_2O_2. At last, slides were counterstained with hematoxylin, dehydrated, and coverslipped. Bex1 staining was assessed under a light microscope (Leica Microsystems, Wetzlar, Germany). Cells with strong or moderate brown staining were rated as positive; cells with no staining were rated as negative, while cells with weak staining were scored separately.

2.6. Double immunofluorescent labeling

After air-dried for 1 h, sections were first blocked with 10% normal donkey serum blocking solution species the same as secondary antibody, containing 3% (w/v) bovine serum albumin (BSA), 0.1% Triton X-100 and 0.05% Tween 20 for 2 h at RT. The sections were then incubated with primary antibodies against Bex1 (mouse, 1:100; Santa Cruz Biotechnology, Santa Cruz, USA), NeuN (rabbit, 1:300; Chemicon, Temecula, CA, USA), glial fibrillary acidic protein (GFAP; rabbit, 1:300; Sigma-Aldrich, St Louis, MO, USA), CD11b (rabbit, 1:500; Abcam), active caspase-3 (rabbit, 1:500; Cell Signaling Technology) overnight at 4 °C, followed by a mixture of FITC- and TRITC-conjugated secondary antibodies (1:1000; Thermo Fisher Scientific, Grand Island, NY, USA) for 2 h at 4 °C. The stained sections were examined with Leica fluorescence microscope (Germany).