Possible Involvement of Caspase-Independent Pathway in Neuronal Death After Subarachnoid Hemorrhage in Mice

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Abstract Early brain injury is now considered as an important cause of delayed neurological deterioration after aneurysmal subarachnoid hemorrhage (SAH), and neuronal apoptosis is one of the constituents of early brain injury. Caspase family is popular proteases in apoptotic pathways, but there also exist caspase-independent cell death pathways in many pathologic states. In this study, we investigated the ratio of caspase-related and caspase-unrelated neuronal deaths in a mice endovascular perforation SAH model. At 24 h after SAH, about half of neurons in the perforation-side cortex showed increased cleaved caspase-3 immunoreactivity. On the other hand, about half of cleaved caspase-3-immunonegative neurons showed abnormal morphology, suggesting that they were in the process of some sort of cell death in the absence of caspase-3 activity. These findings suggest that both caspase-dependent and caspase-independent signaling pathways may cause neuronal death after SAH.

Keywords Cell death · Early brain injury · Neuronal apoptosis · Subarachnoid hemorrhage

Introduction

Delayed neurological deterioration after aneurysmal subarachnoid hemorrhage (SAH) has been a main concern among neurosurgeons for many years, and today it is supposed to be mainly caused by vasospasm and early brain injury (EBI) [1]. Neuronal apoptosis is one of constituents of EBI [1]. Caspase family forms major population among apoptosis-related proteases and works in several apoptotic signaling pathways [5]. Its role in experimental SAH models has already been studied and clarified [4, 7]. However, pan-caspase inhibitors showed great preventive effects against neuronal apoptosis after SAH, but there remained some neurons to be destined to die [7]. Considering that there are some caspase-independent cell death pathways reported in other pathologic states [2, 9], it is supposed that both caspase-dependent and caspase-independent pathways may work in neuronal death after SAH. However, studies of the latter are limited. In this study, thus, we investigated the ratio of immunopositive and immunonegative neurons for cleaved caspase-3, which is an essential protein through apoptotic pathways, and showed the possible involvement of caspase-independent pathways in neuronal death in SAH mice cortex.

Materials and Methods

All procedures were approved by the Animal Ethics Review Committee of Mie University and were carried out in accordance with the institution's Guidelines for Animal Experiments.

SAH Modeling and Study Protocol

C57BL/6 mice (25–30 g, male) were used for SAH modeling or sham operation as previously described [8] (Fig. 1). Briefly, mice were anesthetized, positioned supinely, and skin incision was made at the midline of the neck to expose the left carotid artery. A 4-0 monofilament with a sharpen tip was inserted from the left external carotid artery (ECA) into the left internal carotid artery and push further to perforate the bifurcation of anterior cerebral artery and middle cerebral artery. Then the filament was withdrawn, and the stump

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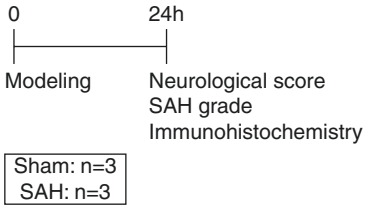

Fig. 1 Experimental designs. Experiment was designed to examine presence or absence of cleaved caspase-3 immunoreactivities among individual neurons in mice cortex after experimental subarachnoid hemorrhage (SAH)

of ECA was coagulated. The wound was sutured. The sham group underwent the same procedure as described above except for perforating the artery. After evaluating neurological scores, mice were sacrificed at 24 h after modeling, and then assessments of SAH grade and immunohistochemistry were performed (Fig. 1). Mice were assigned to SAH ($n = 3$) and sham groups ($n = 3$).

SAH Grade

SAH grade was evaluated as previously described [8]. The basal cistern was divided into six segments, and each segment was allotted a grade from 0 to 3 depending on the amount of SAH. A total score ranging from 0 to 18 was determined by summing the scores. Mice with moderate SAH grade (8–12) were used for experiments as the SAH group.

Neurological Score

Neurological impairments were blindly evaluated as previously described [8]. Neurological scores (3–18) were determined by summing up six test scores (spontaneous activity, spontaneous movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation).

Histology

Mice's brains were used for making paraffin-embedded coronal sections at bregma +1 mm as previously described [8]. At 24 h after modeling, mice were deeply anesthetized with Avertin® (2,2,2-tribromoethanol) solution and perfused with cold phosphate-buffered saline followed by 4% paraformaldehyde for brain fixation. The brains were removed, embedded in paraffin and cut into 4 μm sections. Sections were dewaxed, underwent heat-induced antigen retrieval in 10 mM citrate butter (pH 6.0), and were incubated with rabbit anti-cleaved caspase-3 primary antibody (1:25; Cell Signaling Technology; cat #9661) in SignalStain® antibody diluent (Cell Signaling Technology; cat#8112) at 4 °C for overnight. Then sections underwent reaction with SignalStain® boost immunohistochemistry detection reagent (Cell Signaling Technology; cat#8114) for 30 min at room temperature, visualized by diaminobenzidine (brown color) and counterstained with hematoxylin. Sections were dehydrated, cleared in xylene, and mounted for observation under light microscope.

Cell Counting

Left (perforation-side) temporal base cortex was used for observation of neurons. Neurons were counted in five sequential fields at ×400 magnification. Neurons were determined morphologically as cells having bright, large, and oval nuclei with some prominent nucleoli [6, 10]. Cells with small nuclei were not counted because the possibility of glia was not excluded. Irregular cell outlines, cell shrinkage or pyknosis was defined as abnormal morphology [3].

Statistics

The number of neuron was expressed as means ±standard error of the mean, and unpaired *t*-tests were used for the comparison between the two groups. $P < 0.05$ was considered significant.

Results

Cleaved Caspase-3-Positive Neurons Appeared 24 h After SAH

No mice in the sham group died before sacrifice. Mice in the sham group showed full scores on neurological assessments, while mice in the SAH group showed neurological deterioration at 24 h after operation (data not shown). At 24 h after modeling, there were few neurons being immunoreactive for cleaved caspase-3 in the sham group. On the other hand, about half of neurons showed immunoreactivities for cleaved caspase-3 in the SAH group (Fig. 2). There were significant differences in the number of immunopositive neurons with

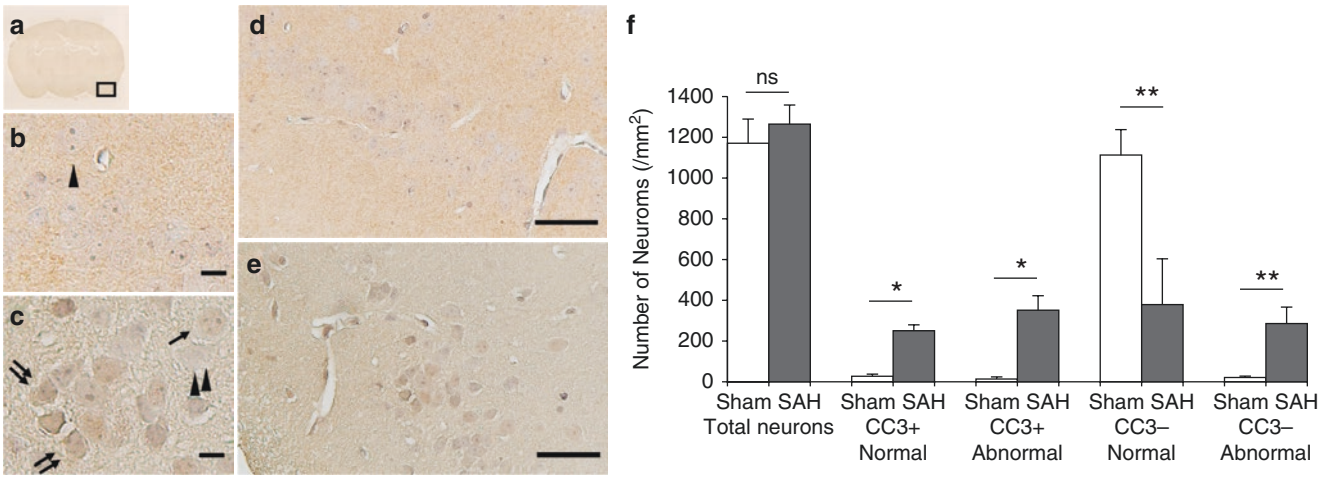

Fig. 2 Cleaved caspase-3 (CC3) immunoreactivity and morphological abnormality in neurons in the ipsilateral (perforation-side) temporal base cortex at 24 h after subarachnoid hemorrhage (SAH) in mice. (**a**) Representative brain coronal section at bregma +1 mm; (**b**–**e**) CC3 immunostaining; (**f**) comparison between the sham and SAH groups. Most neurons are negative for CC3 in the sham group (**b**, **d**), but positive (moderate immunoreactivity) in the SAH group (**c**, **e**). Scale bars: 10 μm (**b**, **c**) and 50 μm (**d**, **e**). Unpaired *t*-test: *ns* no significance; $*P < 0.01$, $**P < 0.05$. Single or double arrows, CC3-positive (CC3+) neurons with normal or abnormal morphology, respectively; single or double arrowheads, CC3-negative (CC3−) neurons with normal or abnormal morphology, respectively. Normal, morphologically normal neurons; abnormal, morphologically abnormal neurons

normal or abnormal morphology between the sham and the SAH groups ($P < 0.01$, respectively, unpaired *t*-test; Fig. 2). There was no significant difference about the total number of neurons between the two groups at 24 h after modeling (unpaired *t*-test, Fig. 2).

Both Caspase-3-Related and Caspase-3-Unrelated Dying Neurons Were Observed After SAH

As to neurons in the SAH group, both positive and negative immunoreactivities for cleaved caspase-3 were observed (Fig. 2). In immunonegative neurons for cleaved caspase-3, normal morphology (i.e., intact neurons) was more frequently observed in the sham group ($P < 0.05$ vs. SAH group, unpaired *t*-test), while abnormal morphology was more frequently observed in the SAH group ($P < 0.05$ vs. sham group, unpaired *t*-test; Fig. 2). About half of immunonegative neurons for cleaved caspae-3 in the SAH group showed morphologically abnormal appearance, which was suggested to be dying.

Discussion

This study showed that almost half of cortical neurons expressed cleaved caspase-3 and that one fourth of cortical neurons had both characteristics of no immunoreactivities for cleaved caspase-3 and morphologically abnormal appearance in the perforation-side temporal base cortex at 24 h after SAH (Fig. 2).

Apoptosis is a well-recognized mechanism under physiological conditions such as developmental or mature stages to form normal organs or eliminate abnormal cells, and also inappropriate triggers make cells inclined to apoptosis under many pathologic states [3]. Caspase family forms major population among apoptosis and inflammation-related proteins [3, 5]. Under physiological conditions, inhibiting caspases sometimes causes cells to undergo necrosis. It is suggested that whether cells are destined to undergo apoptosis or necrosis depends on the extent of adenosine triphosphate depletion and availability of caspases [2]. Nevertheless, under pathologic conditions, caspase inhibitors are commonly considered to have therapeutic effects and direct cells to survive [5]. Among caspase family, caspase-3 works in both extrinsic and intrinsic pathways and is in an essential position in apoptosis signaling [3, 5]. Caspase-3 exists as an inactive precursor and then becomes an active form after cleavage by other proteases [3, 5]. Therefore cleaved (active) caspase-3 could be an indicator of ongoing apoptotic status as used in our study.

Previous studies showed that the rate of active caspase-3-positive cell was higher in SAH mice cortex compared to that in the sham group [4]. In addition, a pan-caspase inhibitor suppressed neuronal apoptosis after experimental SAH [7]. In this study, we compared the number of cleaved caspase-3-positive and caspase-3-negative neurons directly in the temporal base cortex in SAH mice. We defined cell shrinkage, pyknosis, and unusual cell outlines as morphological abnormalities during apoptosis according to previous studies [3, 6, 10]. As a result,

one fourth of neurons had both characteristics of morphological abnormalities and no immunoreactivities for cleaved caspase-3 in mice cortex after SAH. Although there exists the possibility that cells in progressive or terminal stages of caspase-dependent apoptosis had already lost the immunoreactivity for cleaved caspase-3, at least some of them presumably underwent caspase-independent neuronal death.

Apoptosis-inducing factor (AIF) is a molecule released from mitochondrial membrane and known to induce apoptosis directly [2, 9]. In terms of mitochondrial pathways, cytochrome *c*, which is also released from mitochondrial membrane, is thought to have an important role in caspase-dependent pathways through activating caspase-9 [1]. On the other hand, some studies showed that AIF causes apoptosis in a caspase-independent manner [2, 9]. However, its role in SAH models has not been fully investigated. Previous studies suggested that during development, at least partly, caspase-independent pathway can compensate for the lack of caspase activity [9]. Thus, caspase-independent pathways after SAH would be worth investigating in the following two points: (1) finding a new neuronal death pathway in SAH and (2) possible activation of caspase-independent pathways after administrating caspase inhibitors as a potential therapeutic target.

In conclusion, we showed that a considerable number of neuronal deaths were caspase-dependent, but caspase-independent cell death pathways also might be involved in neuronal death after experimental SAH.

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Conflicts of Interest: **We declare that we have no conflict of interest.**

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