Antivasospastic and antiinflammatory effects of caspase inhibitor in experimental subarachnoid hemorrhage

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Object. Inflammation in the subarachnoid space and apoptosis of arterial endothelial cells have been implicated in the development of delayed cerebral vasospasm after subarachnoid hemorrhage (SAH). The authors investigated mechanisms of possible antivasospastic effects of N-benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), a caspase inhibitor that can inhibit both inflammatory and apoptotic systems, in animal models of SAH.

Methods. Rabbits were assigned to three groups of eight animals each and were subjected to SAH by injection of blood into the cisterna magna. The experiments were performed in the following groups: SAH only, SAH + vehicle, and SAH + Z-VAD-FMK. The Z-VAD-FMK (1 mg) or vehicle (5% dimethyl sulfoxide) was intrathecally administered before SAH induction. Diameters of the basilar artery (BA) were measured on angiograms obtained before and 2 days after SAH. The BA diameter on Day 2 was expressed as a percentage of that before SAH. Interleukin (IL–)1β in the cerebrospinal fluid (CSF) was examined using Western blotting, and brains were immunohistochemically examined for caspase-1 and IL-1β. In a separate experiment, 20 rats were subjected to SAH and their brains were immunohistochemically assessed for caspase-1, IL-1β, and macrophages.

Results. In rabbits, Z-VAD-FMK significantly attenuated cerebral vasospasm (the BA diameter on Day 2 in SAH-only, SAH + vehicle, and SAH + Z-VAD-FMK groups was 66.6 ± 3.2%, 66.3 ± 3.7%, and 82.6 ± 4.9% of baseline, respectively), and suppressed IL-1β release into the CSF and also suppressed immunoreactivities of caspase-1 and IL-1β in macrophages infiltrating into the subarachnoid space. Immunoreactivities for caspase-1 and IL-1β were observed in immunohistochemically proven infiltrating macrophages in rats.

Conclusions. These results indicate that caspase activation may be involved in the development of SAH-induced vasospasm through inflammatory reaction. (DOI: 10.3171/JNS-07/07/0128)

Key Words • caspase inhibitor • cerebral vasospasm • inflammation • macrophage • subarachnoid hemorrhage

Delayed cerebral vasospasm with subsequent cerebral ischemia, which usually develops approximately 1 week after SAH, is one of the major causes of morbidity and mortality in patients with ruptured cerebral aneurysms. Despite numerous clinical and experimental studies, the pathophysiological mechanisms underlying cerebral vasospasm have yet to be fully elucidated, and one of the reasons for this is that this peculiar phenomenon seems to be multifactorial. Inflammatory reaction in the subarachnoid space has long been considered to be one of the multiple causative factors. Several lines of evidence have recently been found to support the suggestion that SAH may possibly cause arterial endothelial damage in an apoptotic fashion, eventually triggering or aggravating cerebral vasospasm.

The caspase family is a group of cysteine proteases and has two major properties—as a regulator of proinflammatory and proapoptotic reactions. Caspase-1 is a major proinflammatory caspase and directly converts immature IL-1β into its mature form and is implicated in various inflammatory responses. Caspases such as 3, 8, and 9 play critical roles in the execution of apoptosis. In this study, we used animal models of SAH to investigate mechanisms of a possible antivasospastic effect of an irreversible, broad-spectrum caspase inhibitor, Z-VAD-FMK, which can inhibit both inflammatory and apoptotic systems.

Materials and Methods

All procedures used in this study were performed according to the public health standards at Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.
Caspase activation in cerebral vasospasm

Experiment 1: Rabbit SAH Model

Drug. An irreversible, broad-spectrum inhibitor for caspases, Z-VAD-FMK (Enzyme Systems Products) was dissolved in 5% DMSO prepared with 0.1 mol/L PBS.

Experimental Design of Rabbit SAH Model. Twenty-four male New Zealand White rabbits weighing between 2.0 and 2.3 kg were randomly assigned to one of the following three groups of eight rabbits each and were subjected to experimental SAH as described later. The rabbits in the SAH-only group underwent SAH without any treatment. The rabbits in the SAH + vehicle group received an injection of vehicle (400 μl of 5% [vol/vol] DMSO in PBS). The animals in the SAH + Z-VAD-FMK group received 1.0 mg of Z-VAD-FMK in 400 μl of 5% DMSO. The dosage of Z-VAD-FMK was determined based on the rabbit meningitis model described elsewhere. A vehicle or Z-VAD-FMK injection was administered intrathecally through the cisterna magna 1 minute before SAH induction.

All rabbits underwent vertebralbasilar angiography 5 days before (baseline) and 2 days after (Day 2) the induction of SAH for the evaluation of cerebral vasospasm. On Day 2, 1.0 ml of CSF was withdrawn from the cisterna magna for Western blot analysis and was immediately centrifuged at 6400 G for 3 minutes and stored at −80℃ until use. On Day 2, after assessment procedures were completed, all rabbits were perfusion-fixed with PBS and PBS-containing 4% paraformaldehyde under deep pentobarbital anesthesia. Brains were removed and postfixed overnight in 4% paraformaldehyde at 4℃.

Induction of SAH in Rabbits. The SAH was induced in almost the same fashion as previously described.4 Briefly, rabbits were anesthetized with ketamine (50 mg/kg intramuscularly) and pentobarbital (20 mg/kg intravenously). The atlantooccipital membrane was exposed through an occipitocervical midline incision. After withdrawal of 1 ml CSF, 2.5 ml of fresh autologous arterial blood was injected into the cisterna magna. Thereafter, rabbits were placed in a head-down prone position for 30 minutes.

Cerebral Angiography. Rabbits were again anesthetized with ketamine (50 mg/kg intramuscularly) and pentobarbital (25 mg/kg intravenously) and received mechanical ventilation through an endotracheal tube. A catheter tip was positioned at the origin of the left vertebral artery via the transfemoral route. By injection of 1 ml of contrast medium through the catheter, angiograms were obtained using a digital subtraction angiography system (Advantx/AMF, GE Co.) at the same magnification for each animal. For strict comparison between pre- and post-SAH diameters, a radiopaque marker was placed beneath the rabbit’s head as a reference marker of magnification.

Statistical Analysis. Data for BA diameters in each group were expressed as the mean ± standard deviation, and group differences were tested with analysis of variance followed by a Bonferroni correction. Statistical significance was accepted at a probability value of less than 0.05.

Experiment 2: Rat SAH Model

We evaluated immunohistochemical staining in a rat SAH model, with conventional immunohistochemical and with dual immunofluorescent staining.

Induction of SAH in Rats. Twenty male Sprague–Dawley rats weighing between 350 and 450 g were subjected to SAH under pentobarbital anesthesia (1 mg/kg, intraperitoneally) with basically the same method as in the rabbit SAH model. After withdrawal of 0.1 ml CSF through the atlantooccipital membrane, 0.3 ml autologous arterial blood was injected into the cisterna magna and rats were placed in a head-down prone position for 30 minutes. On Day 2, rats were perfusion-fixed and their brains were postfixed as described in the rabbit SAH model.

Histological Examination of Rats. Ten brains were embedded in paraffin and cut into 4-μm sections. They were then stained with H & E for histological examination under a light microscope.

Immunohistochemical Staining in the Rat SAH Model. The remaining 10 postfixed rat brains were cryoprotected, and frozen sections of these brains were prepared in the same way as in the rabbit model.
Peroxidase immunohistochemistry was performed in the same way as in the rabbit model, by using primary antibodies—goat anti-mouse caspase-1 (dilution ratio 1:400; Santa Cruz Biotechnology) and rabbit anti-rat IL-1β (dilution ratio 1:200; Endogen). Sections were stained with an avidin-biotinylated enzyme complex in which biotinylated secondary antibodies were used as follows: anti–goat and anti–rabbit IgG for caspase-1 and IL-1β, respectively (Vectastain ABC kit, Vector Laboratories). To examine the specificity of the immunoreactivity, some brain sections were processed as described earlier (the primary antibodies were omitted).

Dual Immunofluorescent Staining in the Rat SAH Model. For the determination of cellular localization of caspase-1 and IL-1β in macrophages, dual immunofluorescent staining was conducted for either caspase-1 or IL-1β with macrophages. Sections were initially treated with 10% appropriate normal serum in PBS and then incubated overnight with antibody either for caspase-1 or IL-1β in combination with mouse anti–rat macrophage antibody (dilution ratio 1:200; ED-1, Serotec). The same primary antibodies were used at the same dilution as described earlier in conventional immunohistochemical staining for caspase-1 and IL-1β. These primary antibodies were detected in a mixture of fluorophore-labeled secondary antibodies as follows: Green-labeled Alexa Fluor 488 donkey anti–goat IgG conjugate for caspase-1; Green-labeled Alexa Fluor 488 goat anti–rabbit IgG conjugate for IL-1β; and Red-labeled Alexa Fluor 546 goat anti–mouse IgG conjugate for macrophage staining (Molecular Probes), each at a dilution of 1:200. Sections were coverslipped using mounting medium (Prolong Antifade Kit, Molecular Probes), and the fluorophores were then excited and brought to emission. Sections were viewed under a fluorescence microscope (BX50, Olympus).

Results

Experiment 1: Rabbit SAH Model

Cerebral Angiography in Rabbits. Two days after the induction of SAH, the BA diameters were 66.6 ± 3.16% and 66.3 ± 3.74% (mean ± standard deviation) of the baseline diameter in the SAH-only and SAH + vehicle groups, respectively. In contrast, the diameter was 82.6 ± 4.91% in the SAH + Z-VAD-FMK group, showing that Z-VAD-FMK significantly decreased the magnitude of vasospasm (Figs. 1 and 2).

Western Blot Analysis for IL-1β in Rabbit CSF. Bands with a molecular mass of 17 kD, which corresponded to the mature form of IL-1β, were detected in the CSF from rabbits in the SAH-only and SAH + vehicle groups, but not from animals in the SAH + Z-VAD-FMK group (Fig. 3). The membranes without the primary antibody showed no positive bands (data not shown).

Histological Analysis. The BAs exhibited subintimal thickening and severe corrugation of the internal elastic lamina in the SAH-only and SAH + vehicle groups. These morphological changes were far less pronounced in the SAH + Z-VAD-FMK group (data not shown). Many leukocytes infiltrated into the subarachnoid space in all groups. Many of them were mononuclear cells and some took on a

Fig. 1. Representative angiograms obtained in the rabbit SAH model at baseline (A–C) and on Day 2 (D–F) post-SAH. A and D: Angiograms from rabbits in the SAH-only group. B and E: Angiograms from animals in the SAH + vehicle group. C and F: Angiograms from rabbits in the SAH + Z-VAD-FMK group.
foamlike appearance and were thought to be phagocytosing macrophages (Fig. 4A and B).

**Immunohistochemical Staining for Caspase-1 and IL-1β in the Rabbit SAH Model.** In the SAH-only group, immunoreactivities of caspase-1 (Fig. 5A and B) and IL-1β (Fig. 5C and D) were observed in mononuclear cells infiltrating the subarachnoid clot, which morphologically corresponded to macrophages. The same immunoreactivities were also observed in the SAH + vehicle group (data not shown). Nevertheless, these immunoreactivities were very scarce in the SAH + Z-VAD-FMK group (data not shown). Omission of the primary antibodies resulted in loss of significant immunoreactivities in the brain sections (data not shown).

**Experiment 2: Rat SAH Model**

**Histological Analysis.** Many leukocytes, including mononuclear cells, infiltrated into the subarachnoid space just like in the rabbit SAH model and many BAs exhibited subintimal thickening and corrugation of the internal elastic lamina, which corresponded to vasospasm (data not shown).

**Immunohistochemical Staining for Caspase-1 and IL-1β in the Rat SAH Model.** Immunoreactivity of caspase-1 (Fig. 6A and B) and IL-1β (Fig. 6C and D) were observed in infiltrating mononuclear cells in the subarachnoid clot. Omission of the primary antibodies resulted in loss of significant immunoreactivities in the brain sections (data not shown).

**Dual Immunofluorescent Staining for Caspase-1 and IL-1β in Combination with Macrophages in the Rat SAH Model.** Dual immunofluorescent staining for caspase-1 (Fig. 7A) with macrophage (Fig. 7B), and IL-1β (Fig. 7D) with macrophage (Fig. 7E) verified that caspase-1 and IL-1β were expressed in infiltrating macrophages (Fig. 7C and F).

**Discussion**

The findings in this study are as follows: 1) a caspase inhibitor, Z-VAD-FMK, significantly attenuated cerebral vasospasm in the rabbit SAH model; 2) Western blot analysis confirmed that intrathecal administration of Z-VAD-FMK decreased IL-1β release into the CSF of rabbits affected by SAH; 3) immunoreactivities of caspase-1 and IL-1β were observed in infiltrating mononuclear leukocytes, which morphologically corresponded to macrophages, in the subarachnoid space of rabbits affected by SAH, and these immunoreactivities were suppressed by Z-VAD-FMK; and 4) immunohistochemically proven macrophages infiltrating into the subarachnoid space were immunoreactive for caspase-1 and IL-1β in the rat SAH model. To the best of our knowledge, this is the first immunohistochemical demonstration of the coproduction of caspase-1 and its substrate IL-1β in infiltrating macrophages during SAH.

Of the 14 mammalian caspase family members identified so far,23 caspase-1 is less involved in the apoptotic cascade but is prominent in inflammation or immune responses because of its pivotal role in regulating the cellular export of proinflammatory cytokines.10,33 Caspase-1, formerly known as IL-1β–converting enzyme, directly cleaves inactive precursors of IL-1β and IL-18 into their biologically active forms6,7 and is also reported to play an important role in the production of other proinflammatory cytokines such...
as IL-1α, IL-6, and tumor necrosis factor–α.\textsuperscript{17} The expression of caspase-1 has been observed in various kinds of cells such as monocytes\textsuperscript{17} and macrophages.\textsuperscript{20}

Interleukin-1β is a major regulatory proinflammatory cytokine and a potent inducer for a number of genes, including other cytokines and growth factors, nitric oxide, and prostaglandins.\textsuperscript{6} It is known to be synthesized by a wide variety of cells, including monocytes,\textsuperscript{17} macrophages,\textsuperscript{5} and smooth-muscle cells.\textsuperscript{31} Regarding the direct effect of IL-1β on vessel tone, chronic IL-1β application on arteries is reported to induce smooth-muscle hypercontractility through the upregulation of Rho-kinase.\textsuperscript{14} As a proinflammatory cytokine, IL-1β induces IL-6 expression in fibroblasts\textsuperscript{22} and smooth-muscle cells;\textsuperscript{2} IL-1β–deficient mice exhibited reduced production of IL-6 in the setting of inflammation.\textsuperscript{8} The cytokine IL-1β stimulates endothelial cells to secrete a potent vasoconstrictor (endothelin-1),\textsuperscript{34} is a potent inducer of endothelin receptor expression in vascular smooth-muscle cells, and may be responsible for a generalized activation of the endothelin system.\textsuperscript{21} Interleukin-1β is also a major chemoattractant during inflammation and atherogenesis, inducing chemokines such as IL-8 and adhesion molecules such as intercellular adhesion molecule–1 and vascular cell adhesion molecule–1.\textsuperscript{4,6} Most of the aforementioned IL-
The IL-1β, which is cleaved by caspase-1, and the aforementioned proinflammatory and vasoconstrictive molecules related to IL-1β, have been strongly implicated in the development of SAH-induced vasospasm. The IL-1β is significantly elevated in the CSF of patients with SAH. There is substantial evidence that IL-6, which can cause a long-lasting vasoconstriction, significantly increased in the CSF of patients with SAH and correlated with the incidence of vasospasm. Endothelin-1 has been strongly implicated in vasospasm and its inhibition was reported to ameliorate this condition. Adhesion molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were also reported to be elevated in the CSF of patients with SAH, and intracisternal administration of neutralizing antibody against intercellular adhesion molecule-1 significantly reduced vasospasm in a rabbit SAH model. We have previously reported that the intracisternal administration of decoy oligo-DNA against nuclear factor-κB significantly attenuated vasospasm in a rabbit SAH model.

Thus, considering the aforementioned evidence together with our results on Western blot analysis, which demonstrated that Z-VAD-FMK reduced the release of mature IL-1β into the CSF, the antiinflammatory effect of Z-VAD-FMK might lead to a decrease in the severity of vasospasm, inhibiting caspase-1 and its substrate IL-1β, thereby down-regulating proinflammatory and vasoconstrictive molecules such as IL-6, endothelin-1, and adhesion molecules.

In the present study, we used immunohistochemical assessment to demonstrate the expression of caspase-1 and IL-1β in macrophages infiltrating into the subarachnoid space of rabbits and rats with SAH. In line with our findings, Fassbender et al. showed that coincubation of blood and normal CSF was capable of producing significantly elevated amounts of IL-1β protein, and they also demonstrated that mononuclear leukocytes isolated from bloody CSF in patients with SAH synthesized significantly increased amounts of IL-1β mRNA, concluding that the cellular source of IL-1β in the CSF after SAH is activated mononuclear cells. In our study, Z-VAD-FMK inhibited immunoreactivity of caspase-1 and IL-1β in macrophages infiltrating into the subarachnoid space of rabbits affected by SAH. Therefore, it is conceivable that Z-VAD-FMK attenuated vasospasm by inactivating caspase-1 and subsequently by inhibiting IL-1β production in infiltrating macrophages, and also that infiltrating macrophages may play an important role in the development of vasospasm as a potent producer of various proinflammatory cytokines and vasoconstrictors.

Apoptosis is a process of programmed cell death, in which unnecessary cells are eliminated from multicellular organisms, and is implemented by cascade reactions of the caspase family. Recently Zhou et al. reported that caspase inhibitors attenuated cerebral vasospasm and abolished endothelial apoptosis of cerebral arteries in a dog SAH model, concluding that this antivasospastic effect could result from the inhibition of proapoptotic caspases.
such as 3 and 8 in arterial endothelium. Our study has further shown that the antivasospastic effect of caspase inhibitor could also be related to the antiinflammatory effect in the subarachnoid space.

Several studies have recently demonstrated that the inhibition of caspases reduced the infarction size in animal models of cerebral ischemia, making caspase inhibitors candidates for future antiischemic stroke drugs. Intraventricular administration of caspase inhibitors has been shown to reduce the cerebral infarction size in mice and rats. These reports imply that caspase inhibition could also act protectively against the secondary cerebral ischemia resulting from vasospasm.

Conclusions

We demonstrated that the broad caspase inhibitor Z- VAD-FMK attenuated cerebral vasospasm, with the concomitant suppression of IL-1β release into the CSF, and also with the suppression of immunoreactivities of caspase-1 and IL-1β in macrophages infiltrating into the subarachnoid space in a rabbit SAH model. Taken together with another finding of caspase-1 and IL-1β immunoreactivities in immunohistochemically proven macrophages infiltrating into the subarachnoid space in a rat SAH model, this antivasospastic effect of a caspase inhibitor could be related to the inhibition of caspase-1 and IL-1β in macrophages and the eventual downregulation of IL-1β-related proinflammatory and vasoconstrictive molecules. Given the reports that caspase inhibitors work protectively against cerebral ischemia, caspase inhibition could be a viable strategy not only against SAH-induced vascular narrowing itself but also against cerebral ischemia, which is the detrimental consequence of vasospasm.

References

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Caspase activation in cerebral vasospasm


Accepted January 9, 2007.
Dr. Date holds a grant from the Ministry of Education, Science, Sports, and Culture, Japan; Special Coordination Funds of the Science and Technology Agency of the Japanese government; and Health Sciences Research grants for research on brain science.

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