Subarachnoid hemorrhage (SAH) is associated with high mortality: 12.4% sudden death before receiving medical attention,1 and up to 40% to 60% of patients will die within the first 48 hours because of the initial bleeding.2,3 Major early injuries after the initial bleeding are breakdown of blood–brain barrier (BBB) and formation of brain edema.4 One of the key factors for BBB disruption and brain edema may be apoptotic cell death occurring in neurons5 and in cerebral endothelial cells.6

A large family of cysteine aspartyl protease known as caspases mediates apoptosis after neurological injury.7 Caspase inhibitors prevent apoptosis in a variety of tissues and reduce brain injury in animal models of cerebral ischemia.7 In the present study, we examined the occurrence of apoptosis in brain parenchyma and cerebral endothelial cells after the initial bleeding and tested the possible therapeutic effect of a pan–caspase inhibitor on early brain injury after SAH.

Materials and Methods
This protocol was evaluated and approved by the animal care and use committee at the Louisiana State University Health Sciences Center in Shreveport, Louisiana.

SAH Rat Model
SAH induction was performed as reported previously with slight modifications.9 Briefly, male Sprague-Dawley rats (Harlan, Indianapolis, Ind) weighing 300 to 350 g were anesthetized with α-chloralose (40 mg/kg IP) and urethane (400 mg/kg IP). Animals were intubated, and respiration was maintained with a small animal respirator (Harvard Apparatus). Rectal temperature was maintained at 37±0.5°C with a heating pad. The left external carotid artery was isolated and a 4.0 monofilament nylon suture was inserted through the internal carotid artery to perforate the middle cerebral artery (Figure 1A and 1B). SAH was confirmed at autopsy in each rat. Sham-operated rats underwent the same procedures except that the suture was withdrawn after resistance was felt. Figure 1 demonstrates the anatomy and surgical route of SAH. Brain swelling was apparent immediately after the puncture of middle cerebral artery (Figure 1C). Blood was all over the whole brain at 24 hours after SAH (Figure 1D and 1E).

A broad-spectrum caspase inhibitor, z-VAD-FMK (50 μmol/L per 0.3 mL; Enzyme Systems Products),10–12 was injected intraperitoneally at 1 hour before and 6 hours after SAH induction. z-VAD-FMK suppressed TUNEL and caspase-3 staining in endothelial cells, decreased caspase-3 activation, reduced BBB permeability, relieved vasospasm, abolished brain edema, and improved neurological outcome.

Conclusions—The major effect of z-VAD-FMK on early brain injury after SAH was probably neurovascular protection of cerebral endothelial cells, which results in less damage on BBB. (Stroke. 2004;35:2412-2417.)

Key Words: apoptosis ■ blood–brain barrier ■ brain edema ■ subarachnoid hemorrhage

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2412
The percentage of water content was calculated as [(wet weight – dry weight)/wet weight] × 100%.

**BBB Permeability**

According to the protocol of Uyama et al. and the extraction method by Rossner and Temple, BBB permeability was assessed 24 hours after SAH. Evan's blue dye (2%; 5 mL/kg) was injected over 2 minutes into the right femoral vein and allowed to circulate for 60 minutes. The amount of extravasated Evan's blue dye in the brain was determined by spectrophotometry. Measurements were conducted at excitation wavelength 620 nm, emission wavelength 680 nm, and bandwidth 10 nm.

**Histology**

At 24 hours after SAH, rats were anesthetized and intracardially perfused with physiological buffer solution followed by 4% paraformaldehyde as described previously. Brains were removed and postfixed in 4% paraformaldehyde for 3 days.

For immunohistochemistry, brain sections were incubated overnight at 4°C with primary antibody against cleaved caspase-3 antibody (1:100; Cell Signaling, Beverly, Mass) or primary antibody rabbit anti-matrix metalloproteinase-9 (c-20; 1:200; Santa Cruz Biotechnology, Santa Cruz, Calif.) Sections were then incubated with goat anti-rabbit biotinylated secondary antibody (Santa Cruz Biotechnology) and placed in avidin-biotin-peroxidase complex enzyme. Slides were visualized by incubation with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide. TUNEL staining was conducted according to the protocol of the manufacturer (Roche Diagnostics) as described.

Brain immunolocalization of IgG was studied as described. Briefly, brain slides were incubated with biotinylated goat anti-rat IgG (Santa Cruz Biotechnology) for 2 hours, rinsed in physiological buffer solution, and incubated in avidin-biotin-peroxidase complex for 30 minutes. After rinsing in physiological buffer solution, the reaction product was visualized using 0.05% DAB in the presence of 0.02% H₂O₂.

**Western Blot**

Western blot analysis was performed as described previously. Briefly, brain tissue was homogenized in ice-cold lysis buffer, homogenized, centrifuged, and protein content measured using DC Protein Assay (Bio-Rad). Equal amounts of protein were loaded in each lane of SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. The membrane was blocked with rabbit polyclonal IgG caspase-3 antibody and probed with anti-rabbit IgG-horseradish peroxidase conjugated antibody. Densitometry analysis was performed with the ChemiDoc detection system (Bio-Rad) and Quantity One software (Bio-Rad).

**Statistics**

Data were expressed as mean±SEM. Statistical differences between individual groups were analyzed using 1-way ANOVA followed by Tukey multiple comparison procedure. Significance differences between the groups regarding mortality were analyzed using χ² and z tests (SigmaStat). A P value of <0.05 was considered statistically significant.

**Results**

Mean arterial blood pressure was monitored by a femoral catheter, and blood gas was measured by withdraw blood from femoral artery. z-VAD-FMK did not affect blood pressure or blood gas values (data not shown). A schematic of the experimental design is shown in Figure 2A.

**Mortality**

The mortality at 24 hours after SAH was 42.3% (11 of 26 rats) in SAH+DMSO-treated rats but 25% (4 of 16 rats) in z-VAD-FMK–treated rats (Figure 2A). However, this reduction in mortality after administration of z-VAD-FMK was not significant (P=0.4207; χ²=0.4210; z test). None of the rats died in sham-operated group (0 of 10 rats; Figure 2B).

**Neurological Deficits**

Mean neurological scores of sham-operated, SAH+DMSO, and SAH+z-VAD-FMK groups are compared in Figure 2C. The neurological score of rats with SAH was significantly lower (P<0.05; ANOVA) than sham group at 6 to 12 hours after SAH, and z-VAD-FMK treatment did not alter neurological scores at 6 hours. However, neurological scores improved by z-VAD-FMK at 24 hours after SAH (P>0.05 versus sham).

**BBB Permeability**

SAH produced marked extravasation of Evan’s blue dye into all brain regions at 24 hours, especially in both hemispheres (P<0.05 versus sham; ANOVA; Figure 2D). High values of Evan’s blue dye were obtained in the brain stem and cerebellum, although no statistical significance was obtained (P>0.05 versus sham). Treatment with z-VAD-FMK markedly decreased (P<0.05 versus SAH+DMSO group; ANOVA) the amount of Evan’s blue extravasation in both hemispheres and in brain stem.

**Brain Water Content**

Significant increase (P<0.05) in water content was detected in rats at 24 hours after SAH when compared with sham-operated rats (Figure 2E). z-VAD-FMK decreased water content to a level similar to that of sham-operated group (P<0.05 versus SAH group; P>0.05 versus sham-operated; ANOVA).

**Caspase-3 and TUNEL Staining**

No positive caspase-3 (Figure 3A1 and 3B1) cells were observed in sham-operated rats. At 24 hours after SAH, caspase-3 (p17; cleaved caspase-3)–positive cells were detected in all regions of hippocampus and to a much lesser degree in the cortex (Figure 3A2, hippocampus; 3B2, cortex).
Higher magnification inserts demonstrated positive staining for caspase-3 (Figure 3A2 to 3B2). Similarly, no TUNEL-positive cells were detected in sham-operated rats (Figure 3C1 and 3D1). At 24 hours after SAH, TUNEL-positive cells appeared in the dentate gyrus and CA1 regions of hippocampus (Figure 3C2) and to a lesser degree in basal cortex (Figure 3D2). Treatment with z-VAD-FMK failed to produce remarkable reduction of positive staining either to caspase-3 (Figure 3A3 and 3B3) or to TUNEL (Figure 3C3 and 3D3).

Apoptosis in Basilar Artery Endothelial Cells
SAH produced severe acute vasospasm within 24 hours in the basilar artery (Figure 4B1) when compared with sham-operated (Figure 4A1). z-VAD-FMK prevented cerebral vasospasm after SAH, although the basilar artery was surrounded by blood clots (Figure 4C1). Higher magnification of hematoxylin/eosin staining demonstrated corrugation of internal lamina, contraction of smooth muscle cells, and detachment of endothelial cells, features of cerebral vasospasm (Figure 4B2). In addition, positive staining of caspase-3 and TUNEL was observed in basilar endothelial cells in SAH+DMSO rats (Figure 4E and 4H). No positive staining of caspase-3 or TUNEL was observed in endothelial cells in sham-operated or SAH+z-VAD-FMK animals (Figure 4D, 4G, 4F, and 4I).

Brain IgG Immunostaining
Some tincture in periventricular structures was stained in normal brains (Figure 5A1 to A3), and widespread IgG staining was noted after SAH (Figure 5B1 and 5B2), which means a breakdown of BBB allowing IgG serum proteins to enter the brain parenchyma. A remarkable protective effect from z-VAD-FMK was shown that only weak staining was obtained (Figure 5C1 and 5C2). Higher magnification demonstrated the leaking of IgG in microvessels after SAH (Figure 5B3) and the protective effect of z-VAD-FMK (Figure 5C3).

Caspase-3 Protein Expression
To quantify the inhibitory effect of z-VAD-FMK, protein levels of total caspase-3 (p35) and cleaved caspase-3 (p17) were examined in hippocampus and basal cortex (Figure 6). Quantification showed decreased expression of total caspase-3 but increased cleaved caspase-3 in basal cortex and hippocampus after SAH (P<0.05 versus sham). z-VAD-FMK reduced the expression of cleaved caspase-3 (P<0.05 versus SAH+DMSO).

Discussion
In the present study, we evaluated early brain injury: BBB disruption, brain edema, brain tissue death, as well as mortality and neurological deficits. Cell death, especially apoptosis, occurred in neuronal tissues, particularly in the hippocampus and basal cortex adjacent to the blood deposits. Neuronal cell death contributes to cytotoxic edema, which usually develops early after brain injury. In the meantime, BBB permeability was enhanced as shown by extravasations of Evan’s blue dye, probably because of endothelial apopto-
sis. BBB breakdown leads to vasogenic brain edema after SAH. z-VAD-FMK abolished caspase-3 and TUNEL staining in cerebral endothelial cells and reduced IgG leaking after SAH. Apoptotic death of cerebral endothelial cells might have important impact on neurological functions because z-VAD-FMK improved neurological outcome at 24 hours.

Figure 3. Cleaved caspase-3 (p17)-positive cells were shown in hippocampus (A2) and to a lesser degree in basal cortex (B2) 24 hours after SAH. Higher magnification in the inserts demonstrated the positive staining in the cytosol in CA1, dentate gyrus, and basal cortex. Similarly, TUNEL-positive cells were shown in hippocampus (C2), and to a lesser degree in basal cortex (D2). Higher magnification in the inserts demonstrated the positive staining in the nuclei in CA1, dentate gyrus, and basal cortex. Limited effect of z-VAD-FMK on neuronal apoptosis was observed (A3 to D3).

Figure 4. Normal basilar artery was observed in sham rats (A1 and A2). Severe vasospasm was obtained in the basilar artery surrounded by blood clots in SAH+DMSO rats (B1 and B2). Almost normal basilar artery was surrounded by blood clots in an SAH+z-VAD-FMK rat (C1 and C2). Positive staining of cleaved caspase-3 and TUNEL was observed in cerebral endothelial cells after SAH (E and H, arrows) but not or to a lesser degree in SAH+z-VAD-FMK (F and I, arrow) rats.

After initial bleeding, apoptosis of brain tissues may be caused by elevated intracranial pressure (ICP), toxicity of blood components, ischemia, and reperfusion (reduction and restoration of cerebral blood flow [CBF]), as well as by acute vasospasm. We found apoptotic changes in most brain regions, especially in the hippocampus. The occurrence of...
apoptosis in hippocampus is probably attributable to the use of endovascular filament perforation model, which results in a drastic rise of ICP and reduction of CBF,\textsuperscript{21} resulting in global ischemia, which affects CA1 and dentate gyrus regions of hippocampus.\textsuperscript{22} However, the sporadic occurrence of apoptosis in the cerebral cortex and the weak effect of z-VAD-FMK on caspase-3 and TUNEL staining in neurons demonstrated a strong paradox to the potent effect of z-VAD-FMK on BBB and brain edema.

One of the important observations in the present study is the occurrence of apoptosis in cerebral endothelial cells, which likely contributes to BBB rupture as demonstrated by IgG leakage and Evan’s blue extravasations. Systemic application of z-VAD-FMK decreased endothelial apoptosis, attenuated BBB permeability, and reduced brain edema, probably by its antiapoptotic effect primarily in cerebral endothelial cells. We used a concentration of z-VAD-FMK ($2 \times 50 \mu\text{mol/L}$) systemically, which is comparable with those

![Figure 5.](attachment:image1)

**Figure 5.** Negative staining in sham (A1), strong staining in SAH+DMSO (B1), and weak staining were observed in SAH+z-VAD-FMK rats (C1). Higher magnification demonstrated that IgG leakage occurred mostly around microvessels (B2). Enlarged pictures of IgG leaking from a capillary after SAH (B3) and the effect of z-VAD-FMK (C3) are shown.

![Figure 6.](attachment:image2)

**Figure 6.** Representative bands for total and cleaved caspase-3 are shown for basal cortex and hippocampus at 24 hours after SAH. Total caspase-3 was reduced slightly but significantly ($^*P<0.05$ vs sham), and cleaved caspase-3 was increased ($^*P<0.05$ vs sham). z-VAD-FMK reduced the level of cleaved caspase-3 ($^#P<0.05$ vs SAH+DMSO; $^*P<0.05$ vs sham and SAH+DMSO) without altering the level of total caspase-3 significantly ($n=4$ for each group).
of others using 1 to 100 $\mu$mol/L (or 27 ng to 2.4 mg) in intraventricle or intrathecal injections.\textsuperscript{10} Systemic administration of z-VAD-FMK allows evaluation of its effect, especially on cerebral endothelial cells because z-VAD-FMK does not pass through BBB\textsuperscript{10} and has limited effect on neuronal apoptosis (Figure 3). There is no report about the half life of z-VAD-FMK, and in most studies, only 1 injection of z-VAD-FMK was applied, and the outcome was measured at 24 hours or later.\textsuperscript{10} We administrated z-VAD-FMK at 1 hour before SAH, similar to those of others,\textsuperscript{23} to establish a concept that cerebral endothelial apoptosis may be important for early brain injury after SAH. Post-treatment with potent apoptotic inhibitors is warranted in future studies.

A contradicting issue is the remarkable effect of z-VAD-FMK on BBB permeability and brain edema but weak effect on mortality. This paradox may indicate that, although neurovascular protection is important, other factors such as ischemic cascades or hemoglobin toxicity also matter. The failure of z-VAD-FMK on neuroprotection in hippocampus and cortex regions may underlie, at least partially, its ineffectiveness on mortality. In addition, it is not clear whether z-VAD-FMK might be able to pass through dysfunctional BBB after SAH and offer a limited degree of neuroprotection that may not be detected by the methods used. In conclusion, antiapoptosis could be a new strategy to prevent or attenuate early brain injury after SAH via neurovascular and neuroprotection. More potent and BBB-permeable caspase inhibitors should be tested in future studies.

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References
