Vasospasm and p53-Induced Apoptosis in an Experimental Model of Subarachnoid Hemorrhage

Julian Cahill, MB; John W. Calvert, PhD; Ihsan Solaroglu, MD; John H. Zhang, MD, PhD

Background and Purpose—Despite intensive research efforts, the etiology of vasospasm (sustained constriction of the cerebral vessels) remains unknown. In this study, we investigated the role of p53-induced apoptosis in the vasculature at 24 and 72 hours. To completely examine the apoptotic cascades, key proteins of the caspase-dependent, -independent and mitochondrial pathways were examined.

Methods—In this study, adult rats were divided into 3 groups: sham (n=21), nontreatment (subarachnoid hemorrhage [SAH]+dimethyl sulfoxide; n=42), and treatment (SAH+pifithrin-α) (n=42) groups. Each animal in the SAH group underwent a surgical procedure to induce SAH, and the basilar artery was harvested at 24 and 72 hours for analysis.

Results—We found severe vasospasm at the 24-hour time point, which persisted to 72 hours. Furthermore, we found that the markers of the apoptotic cascades rose significantly at the 24-hour time point but had dissipated by 72 hours. However, the neurological outcome and mortality scores improved at the 72-hour time point.

Conclusions—Apoptosis, and in particular p53, may play an important role in the etiology of vasospasm with relation to SAH, and in this model, vasospasm persisted to 72 hours, despite the fact that apoptosis does not. (Stroke. 2006;37:1868-1874.)

Key Words: apoptosis ■ subarachnoid hemorrhage ■ vasospasm

Subarachnoid hemorrhage (SAH) remains an enigma despite intensive research into the etiology of secondary brain injury as a result of SAH. Two areas of interest have come to the forefront of this research effort, which are early brain injury encompassing the immediate global ischemic injury caused by a fall in cerebral blood flow (CBF) and a rise in intracranial pressure, and vasospasm, which occurs later in the disease process. Of the patients who survive the initial bleed, up to 30% will experience vasospasm over the course of their recovery. Recently, apoptosis in the vasculature has been found to play a significant role in SAH, and it has been suggested that the apoptotic cascades may be responsible, at least in part, for vasospasm. Previously, we have shown that pan–caspase inhibitors attenuated vasospasm in the rat model and that p53 inhibitors may also have a beneficial effect in attenuating vasospasm in the dog model.

As a continuation of this work, we attempted to comprehensively examine the 3 main apoptotic pathways, that being the caspase-dependent and -independent pathways as well as the mitochondrial pathway in relation to vasospasm. p53 is a transcription factor that has been well studied with regard to neoplastic disease in the past. We believe that p53 plays a central organizing role with regard to apoptosis in the vasculature after SAH and that apoptosis may play an important role in the development of vasospasm. The height of apoptosis in the rat coincides with the development of vasospasm and it is known that a rise in intracranial pressure and a fall in CBF leads to a relative, momentary hypoxic state in the brain, which may be the initiating factor for apoptosis to occur in the vasculature leading to vasospasm. To examine the various apoptotic cascades, we chose to examine the roles of cytochrome C as the main protein representing the mitochondrial pathway, apoptosis-inducing factor (AIF) representing the caspase-independent pathway, caspase 8 representing the caspase-dependent pathway, and caspase 3 as an end product of both the caspase-dependent and the mitochondrial pathways.

Materials and Methods
The animal and ethics review committee at Loma Linda University evaluated and approved the protocol used in this study.

Experimental Groups
One hundred forty Sprague-Dawley male rats weighing between 280 and 330 g were randomly assigned to 1 of 3 groups for each time point, 24 and 72 hours: sham (n=21), SAH+dimethyl sulfoxide (DMSO; nontreatment; n=42), and SAH+pifithrin-α (PFT-α; treatment; n=42).

Experimental SAH Rat Model
For this experiment, the rat monofilament puncture model was used, which was originally described by Bederson et al and has been described previously by our laboratory. Briefly, the animals were anesthetized using 4% isoflurane with a mixture of 60% medical air and 40% oxygen, anesthesia was maintained with 2% isoflurane. After intubation, a craniectomy was performed on the contralateral side to the puncture side (left), and a laser Doppler flow probe was glued in place with tissue glue as described previously. CBF was...
then measured by laser Doppler monitor (Periflux system 5000; Perimed). A femoral line was inserted for blood pressure, heart rate, and blood gas analysis. The carotid tree was dissected and a stump made of the external carotid artery. This was used to thread a 4/0 sharpened monofilament suture through the external carotid artery into the internal carotid artery to the junction of the middle and anterior cerebral arteries to create an SAH. A similar procedure was performed in the sham-operated group except that a blunt suture was used and the arterial wall was not penetrated.2

Drug Administration
A p53 inhibitor, PFT-α, was purchased from BIOMOL Inc. PFT-α is a small lipophilic compound that has been shown to cross the blood–brain barrier (BBB). PFT-α was administered at a dose of 2 mg/kg, diluted in DMSO to a final volume of 2 mL, and was administered by intraperitoneal injection 3 hours after the puncture.7 The nontreatment group received DMSO at the same volume.

Mortality and Neurological Scores
The neurological scores were performed in a blinded fashion at 24 and 72 hours and were based on the scoring system of Garcia et al,8 with modifications.3,5 Mortality was calculated at the same time points, as well as the immediate intraoperative mortality.

Western Blot
Western Blot analysis was performed as described by us previously.3,5,9,10 Briefly, basilar artery samples were homogenized, and aliquots of each fraction were used to determine the protein concentration of each sample using a detergent compatible assay (Bio-Rad). Protein samples (50 μg) for p53 and AIF were loaded on an SDS-PAGE gel, electrophoresed, and transferred to a nitrocellulose membrane. Similarly, the protein samples for caspases 3 and 8 and cytochrome C (50 μg) were electrophoresed in Tris Trisine gradient gel.

Membranes were then blocked followed by incubation overnight at 4°C with the primary antibodies. The following primary antibodies were purchased from Santa Cruz Biotechnology: (1) goat polyclonal anti-p53 (C-19), (2) goat polyclonal anti-cytochrome C (C-20), (3) goat polyclonal anti-AIF (D-20), and (4) goat polyclonal anti-caspase 8 (T-16). Finally, rabbit polyclonal anti-caspase 3 was purchased from BD Pharmingen (BD Biosciences). Immunoblots were processed with secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoblots were probed and then exposed to x-ray film. The x-ray films were scanned and the optical density was determined using Image J (NIH).

Histology and Immunohistochemistry
For the histological analysis, each animal was anesthetized, and after perfusion, the brains were removed and postfixed in formalin. Paraaffin-embedded brains were then sectioned and stained with hematoxylin and eosin (H&E) as described previously.9 Immunohistochemistry was performed as described previously4 using the same antibodies used for Western blot. TUNEL staining was performed using a TUNEL staining kit (Roche). The TUNEL-positive cells were expressed by fluorescein dUTP with dNTP or peroxidase with diamino-dianisidine by the manufacturer instructions.10

In addition, the H&E-stained basilar arteries were measured using Image Pro Plus software package (Media Cybernetics). From each animal, 2 basilar artery slices were taken from the proximal, distal, and middle portions of the vessel. Using the software, we calculated the average internal perimeter, minimum, maximum, and mean diameters, as well as the internal area of each slice.

Brain Water Content and BBB
Permeability Assessment
At 24 and 72 hours, the brain water content was measured using the formula (wet weight–dry weight/wet weight)×100%, as described previously.2

The BBB was assessed using the Evans blue dye method, in which 5 mL/kg (2%) Evans blue dye is administered through a femoral line and allowed to circulate for 1 hour before euthanizing the animal. The amount of dye is then measured using a spectrophotometer at an excitation wavelength of 620 nm, emission wavelength of 680 nm, and a bandwidth of 10 nm.3

Data Analysis
Data are expressed as mean±SD. Statistical significance was assured by ANOVA performed in 1-way ANOVA followed by the Tukey test for multiple comparisons. Analysis of physiological variables and arterial measurements were performed by repeated-measures ANOVA. The clinical behavior scores were compared by Kruskal–Wallis 1-way ANOVA followed by multiple comparison procedures by Dunn method. A P value of P<0.05 was considered statistically significant.

Results
Mortality and Neurological Scores
Of a total of 140 rats, 35 (33%) died over the course of the experiment (Figure 1A). No animals in the sham group died. Within the SAH groups, 25% (9 of 35) died either on the table or within the first 3 hours. These animals had not been allocated to a treatment or nontreatment group at that time. From the nontreated group, 48% (17 of 35) died, whereas only 25% (9 of 35) of the treated group died.

The neurological scores revealed a poor score for both treated and untreated SAH animals at 6 hours (14±1 versus 14±1; Figure 1B). However, by 24 hours, the treated rats had a slightly better score (21±1 versus 19±1), and by 48 hours, a significant difference was seen between the 2 groups (25±1 versus 22±1). This difference was maintained at the 72-hour time point (25±1 versus 21±1; P<0.05).

BBB Breakdown and Brain Water Content
The BBB analysis showed a significant protection afforded by PFT-α at the 24-hour time point compared with the DMSO group (3.1±0.3 versus 6.9±0.3; Figure 1C). However, this difference was not evident at 72 hours, when the values in both the treated and nontreated groups returned close to baseline (2.1±0.2 versus 2.5±0.1). These findings were reflected in our investigation of the water content of the brain (Figure 1D). For the brain water content, a significant difference was noted at 24 hours between the treated and nontreated groups (76.7±1.2 versus 79.9±1.6). At 72 hours, there was not a significant difference, although the trend remained (75.6±2.6 versus 76.5±2.4).

Physiological Variables and CBF
Body temperature was maintained throughout the experimental procedure at 37.0±0.5°C. The CBF fell to ≈30% of baseline in the first 5 minutes and rose again to 60% of baseline over the following 2 hours. There was no statistical difference noted between the animals later allocated to the treatment or nontreatment groups (Figure 1E).3,5 Similarly, blood pressure recordings were noted to fall immediately after the puncture and rose again to baseline or slightly above baseline. Blood gases were noted to be similar in all groups, as was the hematocrit.

Western Blot
Western Blot analysis of the basilar artery showed a strong upregulation of p–p53 (5-fold) after SAH, which was markedly inhibited by PFT-α at 24 hours (Figure 2). Although the trend remained at 72 hours, the difference was not as marked. The upregulation of the remaining proteins was not as
striking. Caspase 8 increased after SAH and was attenuated through the inhibition of p53 at the 24-hour time point. Similar results were found for cytochrome C, AIF, and caspase 3. However, the degree of upregulation at the 72-hour time point was not as significant as that found at 24 hours. In addition, with the exception of p53, the apoptotic proteins all rose to a similar degree: ≈1.6-fold.

**Histology**

Representative pictographs of the histological findings can be seen in Figure 3. Severe vasospasm can be seen in Figure 3B. At the higher magnification (Figure 3E), a typical corrugated appearance of the basal lamina is seen, with a thickened and spastic muscular wall. This appearance is very much attenuated in the treated group (Figure 3C). At the 72-hour time point,
much of the blood has disappeared; however, the appearance of the spastic vessel is similar to that observed at 24 hours. By measuring representative pictographs, we found that the mean diameter measurements were 115±11 μm and 195±7 μm for the nontreated and treated groups, respectively, at 24 hours, whereas at 72 hours, values of 122±14 μm and 204±10 μm were observed. The maximum, minimum, and mean diameters are represented in Figure 4A and 4B. The internal area of the basilar artery was also measured, displaying the severity of the vasospasm in the nontreated group (13 848±1440 μm²) compared with the treated group (25 967±1540 μm²) at 24 hours (Figure 4C). At 72 hours, similar values were observed (15 346±1655 μm² and 27 766±1746 μm²) for the nontreated and treated groups. Finally, the internal perimeter of the basilar artery of the nontreated group was 412±40 μm compared with 597±25 μm in the treated group at 24 hours and 443±45 μm
compared with 611±28 μm, respectively, at 72 hours (Figure 4D). The differences observed between the 24- and 72-hour animals were not significant in this study.

Immunohistochemistry
The basilar artery showed TUNEL-positive cells in the endothelial lining of the vessel in the nontreated group at 24 hours (Figure 5B). Although there was some degree of apoptotic cell death evident in the treatment group, it was considerably less (Figure 5C). Staining for p53 and caspase 3 can be seen in the nontreated group in Figure 5E and 5H, respectively. After treatment, not only was the degree of vasospasm alleviated, but the endothelial layer of these vessels showed minimal staining for p53 and caspase 3 (Figure 5F and 5I).

Discussion
Vasospasm remains a leading cause of morbidity and mortality in relation to SAH. Current data suggest a role for Ca\(^{2+}\) channels as a leading cause of vasospasm; however, Ca\(^{2+}\) channel blockers have had a limited impact on the overall morbidity and mortality rates. Therefore, it seems reasonable to suggest that there may be an alternate etiology.

We demonstrated previously that apoptosis occurs in vascular endothelial cells in a patient who died of vasospasm. In this experiment, we evaluated the role of vascular apoptosis in relation to vasospasm in an animal model. Although a direct link between the animal and human models cannot be made at this stage, this experiment lays the groundwork for future studies in humans.

We looked at p53, which we believe is one of the main orchestrating proteins in relation to apoptosis. We have previously shown that p53 is stabilized in the cytoplasm by tumor necrosis factor-α, which acts via a death receptor present in the cell membrane. The phosphorylation and subsequent stabilization of p53 in the cytosol are important because they allow for the initiation of the apoptotic cascades.

p53 is believed to act through the BCL2 family of proteins, which, in turn, controls the release of cytochrome C from the mitochondria. The release of cytochrome C results in the creation of the apoptosome, which cleaves procaspase 3 to form caspase 3. Caspase 3 has been shown to be the end product for both the external and internal apoptotic cascades. Caspase 8 can directly influence caspase 3 through the caspase-dependent pathway. Finally, p53 has also been shown to result in the release of AIF from the mitochondria, which represents the caspase-independent pathway. We demonstrated a significant reduction in these proteins as a result of the inhibition of p53 alone. This resulted in an overall dampening of the apoptotic process. Furthermore, it seems to suggest that these cascades are to some degree dependent on p53.

By reducing apoptosis in the basilar artery, we were able to prevent severe vasospasm from occurring at both the 24- and 72-hour time points. The overall reduction in vasospasm was significant as seen by the basilar artery measurements (Figure 4). A possible mechanism for this relates to the ability of the endothelial cells to prevent smooth muscle cell proliferation and vasoconstriction by producing inhibiting factors such as...
endothelial NO synthase. To date, the long-term consequences of vasospasm in this experimental model have not been considered. Although the time course for apoptosis in this model is ∼24 hours, we found that upregulation of the apoptotic machinery was still evident at 72 hours on Western blot analysis. Apoptotic markers were not seen at 72 hours using immunohistochemical techniques (data not shown). Indeed, this fact was reflected in our measurements of outcome, BBB breakdown, and brain edema, all of which subsided at 72 hours. This seems to suggest that the preven-

Figure 4. Basilar artery measurements at 24 and 72 hours. The mean, maximum, and minimum diameters are shown in A and B. The internal area of the basilar artery (C) was also measured, displaying the severity of the vasospasm in the nontreated group. Finally, the internal perimeter of the basilar artery is shown in D. The differences observed between the 24- and 72-hour animals were not significant in this study. *P<0.05 compared with sham; **P<0.05 compared with nontreatment and sham; #P<0.05 compared with nontreatment but not sham (ANOVA with Tukey test).

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Figure 5. Immunohistochemistry. TUNEL staining is shown in A through C, which demonstrates positive staining (arrows) in the nontreatment group (B) compared with the treated group (C). Staining for p53 is shown in D through F, where there is minimum staining in the treated group (F). Finally, caspase 3 immunohistochemistry is shown in G through I, demonstrating again the minimal degree of staining in the treated group (I). Arrows denote positively staining cells. Bars=200 μm.
tion of p53-orchestrated apoptosis in the endothelial layer preserves the integrity of the vasculature, thereby protecting the BBB and preventing edema. However, it must be pointed out that the prevention of apoptosis may have had a direct influence on the BBB itself, which may also have led to an overall improvement. This will be the focus of future studies in this area.

Overall, the findings of this article seem to suggest that clinically, the height of the injury occurs at 24 hours, which can be attenuated by inhibiting p53, thereby reflecting its central role with regard to the apoptotic cascades in SAH. Although the vasospastic appearance of the basilar artery is still evident at 72 hours, we did not see poor neurological scores or evidence of BBB disruption. This suggests that although apoptosis in the endothelial cells begins to subside at 72 hours, smooth muscle proliferation in the vessel is not affected. In fact, previous studies have shown that vasospastic vessels can be seen as far out as 5 days. Therefore, we cannot attribute a reduction in apoptosis to the improvements seen at 72 hours. Whether or not it can be surmised from these data that vasospasm may not be as important with regard to brain injury after SAH as believed previously requires additional experimental data.

Furthermore, the inhibition of p53 did not result in complete attenuation of vasospasm, suggesting that the complete inhibition of apoptosis would require multiple inhibitors. Therefore, inhibition at multiple levels may provide better protection from vasospasm for patients in the future and presents us with an exciting therapeutic option deserving of further work.

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Disclosures

None.

References


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