EMOGLOBIN appears to play an important role in the pathogenesis of vasospasm after SAH. Modification of the metabolism of hemoglobin and heme may be important in the prevention of vasospasm. Oxidation of the ferrous iron in oxy- or deoxyhemoglobin produces methemoglobin, which more readily releases its heme groups from the globin chains. Globin chains are probably degraded by intra- and extracellular proteases. The heme group is metabolized into biliverdin, CO, and iron by HOs that consist of at least three isozymes: the oxidative stress-inducible protein HO-1, constitutively expressed HO-2, and HO-3. Heme oxygenase-1 (also known as heat shock protein 32) usually is not expressed except in response to stimuli such as hemin, heavy metals including iron, ultraviolet light, H$_2$O$_2$, sodium arsenite, lipopolysaccharide, and heat shock in rats but not in humans. In contrast, HO-2 and HO-3 are constitutively expressed in many cell types, including neurons. Induction of HO-1 after SAH in rats was suggested to have neuroprotective effects in the brain, and inhibition of HO-1 expression in these arteries was reported to increase vasospasm in cerebral arteries. It has never been directly demonstrated, however, whether induction of HO-1 may be used to modify cerebral vasospasm. In these experiments we present direct evidence of a vasodilatory contribution of the HO-1–CO system in the major cerebral arteries of rats, as well as evidence that overexpression of HO-1 in cerebral arteries can reduce vasospasm after experimental SAH.

Object. Hemoglobin causes contraction of cerebral arteries and is also believed to cause vasospasm after subarachnoid hemorrhage (SAH). The goal in this study was to determine if overexpression of heme oxygenase-1 (HO-1), the principal enzyme involved in the metabolism of hemoglobin, would reduce contractions of cerebral arteries brought on by hemoglobin and decrease vasospasm after experimental SAH.

Methods. Injection of adenovirus expressing HO-1 (Ad5HO-1) into the cisterna magna of rats produced a significant increase in expression of HO-1 messenger RNA, and protein and HO-1 activity in the basilar artery (BA; p < 0.05 for each measure compared with vehicle and/or control virus, according to analysis of variance or unpaired t-test). Injection of adenovirus expressing β-galactosidase (Ad-βGal) produced only mild, statistically nonsignificant increases. The HO-1 immunoreactivity was localized to the BA adventitia after injection of Ad5HO-1 or Ad-βGal. Injection of Ad5HO-1 and Ad-βGal increased the baseline diameter of the BA (measured directly via a transclival window) and brainstem cerebral blood flow (CBF), measured by laser Doppler flowmetry, compared with vehicle. Contraction of the BA after addition of hemoglobin was significantly inhibited, reduction in brainstem CBF was significantly prevented, and carboxyhemoglobin concentration was significantly increased in rats injected with Ad5HO-1 compared with Ad-βGal and vehicle. Vasospasm was significantly ameliorated in rats in which Ad5HO-1 was injected into the cisterna magna at the time of SAH in a double-hemorrhage model.

Conclusions. These results show that overexpression of HO-1 inhibits arterial contractions induced by hemoglobin and can reduce vasospasm after experimental SAH.

KEY WORDS • subarachnoid hemorrhage • vasospasm • basilar artery • hemoglobin • adenovirus • rat

H. J. Neurosurg. / Volume 96 / June, 2002

Abbreviations used in this paper: ANOVA = analysis of variance; BA = basilar artery; CBF = cerebral blood flow; CMV = cytomegalovirus; CO = carbon monoxide; CSF = cerebrospinal fluid; HO = heme oxygenase; LDF = laser Doppler flowmetry; mRNA = messenger RNA; NOS = nitric oxide synthase; PBS = phosphate-buffered saline; pfu = plaque-forming units; RT-PCR = reverse transcriptase–polymerase chain reaction; SAH = subarachnoid hemorrhage.
Prevention of vasospasm with HO-1 gene therapy

José A. Maldonado, M.D.,* Alan P. Malden, M.D.,† A. Ernesto Anzaldua, M.D.,* and Datto M. Kasem, M.D., Ph.D.*

J. Neurosurg. / Volume 96 / June, 2002

A double-hemorrhage model of SAH was used to assess whether adenovirus-mediated HO-1 gene transfection prevents vasospasm in vivo. Male Sprague–Dawley rats were randomly assigned to three groups to receive intracisternal Ad5HO-1, Ad-βGal, or vehicle. The investigator performing injections and analyzing data did so in a blinded fashion. On Day 0, animals were anesthetized and allowed to breathe spontaneously, after which 0.25 ml of arterial blood was mixed with 0.1 ml of Ad5HO-1, Ad-βGal (10^10 pfu), or vehicle and injected into the cisterna magna over 5 minutes. Rats were reanesthetized 2 days after the initial injection (Day 2) and given a second injection of 0.3 ml of autologous arterial blood. Seven days after the first injection, rats were killed with overdoses of anesthetic agents and their tissues were harvested, or the animals were fixed by perfusion with PBS, followed by 4% paraformaldehyde in PBS at physiological blood pressure. Frozen sections of BA and brainstem were cut 10 μm thick on the cryostat, and BA diameters were measured using a light microscope equipped with a micrometer. Cross-sections of BA were obtained for measurement at three points: 200 μm above the union of the vertebral arteries, just below the anterior or inferior cerebellar arteries, and 200 μm below the BA bifurcation. The mean of the three points was used as the diameter of the BA.

Data Analysis
All data analysis was conducted in a blinded fashion, and data are expressed as the mean ± standard deviation. Comparisons of levels of mRNA and protein, and arterial diameter and blood flow within and between groups, were made using ANOVA for multiple comparisons, followed by pairwise comparisons with the Fisher test if significant variance was found. Paired or unpaired t-tests were used for comparisons between two measurements. A probability value of less than 0.05 was considered statistically significant.

Sources of Supplies and Equipment
The charge-coupled device camera and video monitor were purchased from Hitachi, Yokohama, Japan. The optical measuring device and the micrometer-equipped light microscope were acquired from Fisher Scientific, Pittsburgh, PA. The blood pressure monitoring device was obtained from Stoelting, Wood Dale, IL, and the PaO2 and PaCO2, monitoring device (i-STAT System) was obtained from Sensor Devices, Waukesha, WI. The purified hemoglobin was supplied by Hemosol, Etobicoke, ON. The LDF device was purchased from Transonic Systems, Ithaca, NY. The Phosphor Imager and Image Quant software were acquired from Molecular Dynamics, Sunnyvale, CA. The rabbit polyclonal antibody against rat HO-1 was obtained from R&D Systems, Minneapolis, MN. The BCA protein assay kit was obtained from Pierce Chemical Co., Rockford, IL. The spectrophotometric device (Spectronic Genesys 5) was obtained from Milton Roy, Ivyland, PA.

Results
Expression of Recombinant HO-1 Adenovirus in the BA
The BAs that were removed 1 day after injection of Ad5HO-1 into the cisterna magna contained increased amounts of HO-1 mRNA (Fig. 1), whereas injection of Ad-
Gal or vehicle (10% glycerol in saline) did not result in substantial expression of HO-1 mRNA (three rats per group). There was a statistically nonsignificant increase in expression of HO-1 mRNA in the BAs of rats injected with Ad-Gal that was significantly less than in the BAs of rats injected with Ad5HO-1 (p < 0.001, ANOVA). Significantly increased HO-1 protein, as determined on immunoblot analysis, also was observed in the BA 1 day after intracisternal injection of Ad5HO-1 (three rats per group, p < 0.001 compared with other groups; ANOVA). Injection of Ad-Gal resulted in a low level of expression of HO-1 protein in the BA, whereas injection of vehicle did not.

Immunohistochemical studies of BAs obtained 1 day after injection of Ad5HO-1, Ad-Gal, or vehicle showed negligible HO-1 immunoreactivity after injection of vehicle (three rats per group, Fig. 3). Marked HO-1 immunoreactivity was detected in the rat BA 1 day after Ad5HO-1 injection. Immunoreactivity was confined to numerous cells in the adventitia and to inflammatory cells that had surrounded the BA and that were not seen after injection of vehicle or around normal BAs. There was no detectable staining in the smooth-muscle cells of the tunica media or in the tunica intima. Occasional cells showing immunoreactivity for HO-1 were observed in the adventitia of BAs of rats injected with Ad-Gal, although these cells were much less abundant than after injection of Ad5HO-1. Inflammatory cells containing HO-1 immunoreactivity also were seen around the BA of rats injected with Ad-Gal. There was no qualitative difference in the amount of inflammation observed after injection of Ad5HO-1 or Ad-Gal.
Prevention of vasospasm with HO-1 gene therapy

Effect of HO-1 Transfection on BA Diameter and CBF

The baseline diameter of the BA and its diameter after exposure to increasing doses of ferrous hemoglobin were examined 1 day after intracisternal injection of Ad5HO-1 (10^9 pfu, five rats), Ad-βGal (10^9 pfu, five rats), or an equivalent volume of vehicle (six rats). Physiological parameters were maintained within the normal range (Table 1). The diameter of the BA at baseline was significantly greater in the Ad5HO-1 (423 ± 100 μm) and Ad-βGal (419 ± 13 μm) groups compared with vehicle (367 ± 11 μm, p < 0.001, ANOVA, Fig. 4), although there was no significant difference in diameter between the Ad5HO-1 and Ad-βGal groups. Application of 10^{-7} to 10^{-4} mol/L of pure hemoglobin led to contractions that started within minutes and that reached a maximum in 10 minutes. Washout of the hemoglobin with PBS reversed the contraction to baseline diameter in 50 minutes. Hemoglobin caused a concentration-dependent contraction of the BA in each group. To allow for differences in the baseline diameter between groups, the contractions to hemoglobin were expressed as a percentage of the baseline diameter. This did not change the overall pattern, however, which was that contractions of arteries from animals transfected with Ad5HO-1 were always less. The maximal percentage of contraction (Fig. 4) was significantly less (p < 0.001, ANOVA; Fig. 5). For 10^{-4} mol/L hemoglobin, the CBF was significantly higher in the Ad5HO-1 group compared with the other groups (p < 0.001, ANOVA).

Activity of HO and Carboxyhemoglobin Formation

To determine whether adenovirus-mediated HO-1 transfection produces active HO protein and CO, we examined HO activity and the carboxyhemoglobin concentration in untreated control rats (four for each factor) and in rats 1 day after injection of Ad5HO-1 (six rats each), Ad-βGal (HO activity in six and carboxyhemoglobin in four), or vehicle (HO activity in five and carboxyhemoglobin in three) into the cisterna magna. The HO activity was increased significantly in the Ad5HO-1 group compared with the vehicle.

**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad5HO-1 (5 rats)</td>
<td>Ad-βGal (5 rats)</td>
<td>Vehicle (6 rats)</td>
</tr>
<tr>
<td>blood pressure (mm Hg)</td>
<td>132 ± 13</td>
<td>127 ± 16</td>
<td>130 ± 17</td>
</tr>
<tr>
<td>PaCO₂ (mm Hg)</td>
<td>39 ± 2</td>
<td>42 ± 1</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td>88 ± 5</td>
<td>87 ± 5</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>pH</td>
<td>7.40 ± 0.02</td>
<td>7.41 ± 0.02</td>
<td>7.41 ± 0.06</td>
</tr>
</tbody>
</table>

* All values are expressed as the means ± standard deviations.
Vehicle and untreated control groups (p < 0.001, ANOVA; Fig. 6). The HO activity was also higher in the Ad5HO-1 than in the Ad-βGal group, although this was significant only according to the unpaired t-test (p < 0.05). Similarly, for carboxyhemoglobin, there was significant variance, with Ad5HO-1 treatment associated with significantly higher concentrations than in the vehicle or untreated control groups (p < 0.001, ANOVA) and significantly higher concentrations than in the Ad-βGal–injected group according to the unpaired t-test (p < 0.05, Fig. 6).

Effect of Ad5HO-1 on Vasospasm After SAH

Five of 24 rats died of respiratory arrest after the first or second cisternal blood injection. The 19 surviving rats (Ad5HO-1, six rats; Ad-βGal, six rats; vehicle, seven rats) exhibited normal behavior and appetite after recovery from SAH and anesthesia. Two additional rats, both in the vehicle group, were killed before Day 7 because of dehydration, anorexia, or severe decrease in activity. Expression of HO-1 mRNA in the Ad5HO-1–injected group increased significantly 7 days after SAH compared with Ad-βGal– or vehicle–injected groups (Fig. 7). Immunoblotting showed that expression of HO-1 protein was significantly greater in the Ad5HO-1 group compared with the Ad-βGal and vehicle groups on Day 7 (p < 0.05, ANOVA). Although HO-1 mRNA was detected on vehicle-injected rats with SAH, protein was not detected by immunoblotting with methods that detected protein in the virus-injected groups.

Overexpression of HO-1 by cisternal injection of Ad5HO-1 significantly diminished cerebral vasospasm after SAH (Fig. 8). The diameter of the BA 7 days after SAH was significantly greater after injection of Ad5HO-1 (380 ± 36 μm) compared with Ad-βGal (282 ± 44 μm) and vehicle (287 ± 31 μm, p < 0.001; ANOVA).

Discussion

The new findings in this experiment are that rat BA diameter and CBF can be increased by transfection of cells in the adventitia with adenovirus expressing HO-1 or β-galactosidase. Overexpression of the HO-1 protein in the BA of rats specifically increases HO activity, diminishes contractions of the artery to hemoglobin, prevents the reduction in blood flow that occurs after exposure to hemoglobin, and decreases vasospasm after SAH.

Previous investigations have shown that injection of

S. Ono, T. Komuro, and R. L. Macdonald

FIG. 4. A: Bar graph of baseline BA diameter 1 day after injection of Ad5HO-1 (five rats), Ad-βGal (five rats), or vehicle (six rats), showing significantly increased diameter after injection of either virus compared with vehicle (*p < 0.001 according to ANOVA). B: Graph showing concentration-contraction curves generated by application of pure hemoglobin to the BA of rats in which Ad5HO-1, Ad-βGal, or vehicle was injected into the cisterna magna 1 day earlier. Data are expressed as the percentage of change from baseline. Injection of Ad5HO-1 was associated with less contraction to hemoglobin (10⁻³–10⁻⁴ mol/L hemoglobin, p < 0.05 according to ANOVA) compared with the other groups.

FIG. 5. Graph showing baseline LDF-measured blood flow in the brainstem 1 day after injection of Ad5HO-1 (five rats), Ad-βGal (five rats), or vehicle (six rats), and after application of 10⁻³ to 10⁻⁴ mol/L hemoglobin. Baseline flow and flow after application of hemoglobin were significantly higher in both groups injected with virus than in the vehicle-injected control animals for all concentrations except 10⁻⁴ mol/L hemoglobin in animals injected with Ad-βGal (*p < 0.01 according to ANOVA). For 10⁻⁴ mol/L hemoglobin, flow was significantly higher in the Ad5HO-1 group compared with the other groups (*p < 0.001 according to ANOVA).
Prevention of vasospasm with HO-1 gene therapy

Fig. 6. Bar graphs showing HO activity (A) and carboxyhemoglobin (CO-hemoglobin) concentration (B) in rats that 1 day earlier had received intracisternal injections of Ad5HO-1 (six rats), Ad-βGal (HO activity, six rats; carboxyhemoglobin, four rats), or vehicle (HO activity, five rats; carboxyhemoglobin, three rats), or untreated control rats (HO activity and carboxyhemoglobin, four rats each). The HO activity was increased significantly in the Ad5HO-1 group compared with the vehicle and untreated control groups (*p < 0.001 according to ANOVA) and compared with the Ad-βGal group (*p < 0.05 according to the unpaired t-test). Carboxyhemoglobin concentrations showed significant variance, with Ad5HO-1 associated with significantly higher concentrations than vehicle or untreated controls (*p < 0.001 according to ANOVA), and significantly higher concentrations than Ad-βGal, according to the unpaired t-test (p < 0.05).

Fig. 7. A: Representative blots showing RT-PCR amplification of rat HO-1 (upper blot) and β-actin (lower blot) mRNA from BAs in rats with SAH and injection of Ad5HO-1 (first three lanes), Ad-βGal (fourth and fifth lanes), or vehicle (lanes 6–8) into the cisterna magna 7 days earlier. There is expression of HO-1 mRNA after injection of Ad5HO-1 and Ad-βGal. B: Bar graph showing semiquantification of RT-PCR bands for each group (three rats per bar). There is significantly more HO-1 mRNA after injection of Ad5HO-1 compared with the other groups (p < 0.01 according to ANOVA).

function when the arteries were removed and studied under isometric tension in vitro.17,8,51 In most of these studies vascular function, arterial diameters at baseline, or CBF in vivo were not assessed.

We previously reported that injection of adenovirus expressing endothelial NOS increased the baseline BA diameter in dogs;40 arterial diameter was assessed 7 days after viral injection. In the present study we have shown that injection of both control adenovirus expressing β-galactosidase and of Ad5HO-1 increased BA diameter and brainstem CBF 1 day later. This may be because injection of virus was associated with acute inflammation, which is known to increase CBF acutely.15,25 The increase in CBF may be due to expression of inducible NOS in inflammatory cells, with vasodilation due to the production of nitric oxide.16 The finding that inhibition of post-SAH inflammation by intracisternal administration of antisense oligonu-
cleotides to nuclear factor-κB prevented vascular contraction 7 days after SAH against this mechanism. Another explanation is that both viruses were associated with HO-1 expression and that CO produced by HO-1 led to vasodilation; there is evidence that CO dilates systemic arteries. It has been reported that CO increased CBF in rats, although this phenomenon also could be due to the reduced O$_2$-carrying capacity of carboxyhemoglobin. Brian, et al., reported that CO did not dilate cerebral arteries, but this finding is at variance with the accepted opinion that CO is a vasodilator.

It is unlikely that HO-1 expression was a nonspecific response to the inflammation in the AdSHO-1 group, because the virus is known to produce HO-1 in rats, and there was greater HO-1 expression after injection of this virus than after injection of Ad-βGal. It must be acknowledged, however, that we cannot differentiate endogenous HO-1 from HO-1 produced by the virus, because both were of rat origin. On the other hand, HO-1 may suppress inflammation, so it would be expected that nonspecific induction of HO-1 due to inflammation would be less in this group. We observed no differences in the degree of inflammation associated with each virus. In our previous study we did not find dilation of the BA in dogs 7 days after intracisternal injection of Ad-βGal. One possibility is that the dilatory effects of inflammation induced by Ad-βGal are transient. The CBF is elevated in the acute stages of meningitis, and when inflammation becomes chronic the CBF is reduced. Furthermore, inhibition of inflammation 7 days after SAH reduced vasospasm. The duration of transgene expression also must be taken into account, although it was not investigated fully in this model. Christenson, et al., noted that transgene expression lasted only a few days when it was driven by the CMV promoter. There was increased HO-1 expression 7 days post-SAH, after injection of AdSHO-1, although this did not appear to be as high as after 1 day.

Production of HO-1 in cells in the adventitia was associated with an increase in diameter of the BA and with an increase in CBF in the adjacent brainstem. The vasodilation could be due to several mechanisms. The CO produced by HO-1 could diffuse from adventitial cells to the smooth-muscle cells to mediate this effect. This also would be consistent with the selective reduction in hemoglobin-induced contraction of the BA and the increase in brainstem CBF that was observed. Expression was achieved only in the arterial adventitia, which is consistent with previous reports on the results of cisternal injection of viruses.

We previously reported that hemoglobin mediates vasospasm; numerous mechanisms have been proposed, but most involve the intact hemoglobin molecule. Thus, a method for removing hemoglobin more rapidly from the subarachnoid space after SAH might be efficacious in preventing vasospasm. These experiments show that overexpression of HO-1 can reduce the contractile effect of hemoglobin. Numerous mechanisms are possible: overproduction of CO could mediate vascular relaxation or could bind to hemoglobin, preventing hemoglobin from scavenging nitric oxide. The HO could metabolize hemoglobin, producing more CO, which would have the same effects, and reducing the amount of vasoactive hemoglobin. It has been shown previously that induction of HO-1 may mediate similar effects in the systemic vasculature. Infusion of hemoglobin in rats usually elevates blood pressure although this does not occur 1 day after animals are stressed and at a time when HO-1 is induced in the vasculature. Induction of HO-1 is often associated with increased expression of ferritin. We did not assess ferritin levels but iron uptake by ferritin could theoretically be involved in preventing hemoglobin-related contraction, because iron compounds may contract cerebral arteries. Iron chelators prevent experimental vasospasm but it seems unlikely that iron released from hemoglobin would participate in its acute contractile action.

After observing reduced contractions to hemoglobin, we tested whether Ad5HO-1 could prevent vasospasm after SAH. The rat model of SAH was chosen, which has the benefits of simplicity, economy, and production of a delayed arterial narrowing that lasts for up to 7 days. Two injections of blood were administered, because it is widely accepted that just one injection produces vasospasm that lasts for only 2 days and that is not associated with other accepted features of vasospasm, such as pathological changes in the cerebral arteries. Overexpression of HO-1 reduced vasospasm after SAH. In addition to the mechanisms discussed earlier, HO metabolism of heme produces biliverdin, which could be converted to bilirubin. The importance of this compound in vasospasm is uncertain because it has been reported to cause vasospasm, but it also is an antioxidant that could reduce oxidative reactions that are suggested to mediate vasospasm. Our finding that expression of HO-1 reduces vasospasm is consistent with earlier reports of a role for HO-1 in vasospasm.

We have reported that HO-1 and ferritin are increased in monkey cerebral arteries 7 and 14 days after SAH, at a time when vasospasm is resolving even in the continued presence of SAH, indicating that there may be an adaptive response of the cerebral arteries that allows them to relax in the continued presence of the subarachnoid blood that caused the spasm in the first place. Suzuki, et al., showed that HO-1 was markedly overexpressed in the rat BA after SAH and that inhibition of HO-1 expression increased va-

![Fig. 8. Bar graph of BA diameter 7 days after SAH in a double-injection model with injection of Ad5HO-1 (five rats), Ad-βGal (five rats), or vehicle (six rats) at the time of initial SAH, showing significantly increased diameter after injection of either virus compared with vehicle (p < 0.001 according to ANOVA).](image-url)
Prevention of vasospasm with HO-1 gene therapy

vasospasm and delayed clearance of hemoglobin from the subarachnoid space after SAH. Theoretically, an additional beneficial effect is that HO may be neuroprotective in cerebral ischemia which is the mechanism by which vasospasm would damage the brain.\footnote{10,19,49} We could not assess neurological effects of vasospasm in this model because ischemia is not produced.

Conclusions

We report successful transfection of the arterial adventitia of the rat BA with HO-1. Although adenoviral transfection is associated with a nonspecific increase in vascular diameter and CBF after 1 day, HO-1 overexpression specifically prevents hemoglobin-induced contraction of the BA and vasospasm after SAH in a rat double-hemorrhage model. Further investigations are needed to determine the mechanisms of these effects and whether vasospasm can be prevented in models of this disease in large animals.

Acknowledgments

We thank Hemosol for providing pure hemoglobin, Drs. A. Choi for supplying AdSHO-1, and J. Schaak for supplying Ad-βGal.

References


Manuscript received August 13, 2001. Accepted in final form January 15, 2002. This study was supported by Grant No. NS01831 to Dr. Macdonald from the National Institutes of Health and the Brain Research Foundation.

Address reprint requests to: R. Loch Macdonald, M.D., Ph.D., Section of Neurosurgery, MC3026, University of Chicago Medical Center, 5841 South Maryland Avenue, Chicago, Illinois 60637. email: lmacdona@surgery.bsd.uchicago.edu.
Title