The role of hemoglobin in the etiology of cerebral vasospasm

An in vivo study of baboons


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Oxyhemoglobin was injected intracisternally into three baboons, and methemoglobin into one baboon, in an attempt to mimic the prolonged cerebral arterial spasm sometimes seen after subarachnoid hemorrhage due to aneurysm rupture. Cerebral angiography was performed for up to 7 days after injection of hemoglobin, and the degree of vasospasm was estimated from the angiograms. Oxyhemoglobin caused slight arterial narrowing, which lasted for 3 days. Methemoglobin had no significant effects. Motor neurological deficits and histopathological signs, characteristic of prolonged cerebral vasospasm, were not observed. It was concluded that hemoglobin alone is not capable of causing the cerebral vasospasm syndrome in these experimental animals.

KEY WORDS • subarachnoid hemorrhage • oxyhemoglobin • cerebral vasospasm • methemoglobin

Cerebral arterial spasm is a major complication in the treatment of subarachnoid hemorrhage (SAH) due to aneurysm rupture. Prolonged arterial narrowing as demonstrated by angiography is usually maximal 5 to 10 days after release of blood into the subarachnoid space. In cases of severe spasm, the resultant reduction in cerebral blood flow may lead to ischemia in the territories of the arteries affected, and concomitant neurological deficits. Despite much investigation, the etiology of cerebral vasospasm remains obscure, and a rational chemotherapy for this disorder cannot be formulated. Recent work from our laboratories has implicated vasconstrictor substances derived from blood and damaged tissues in the genesis of delayed cerebral arterial spasm. Although we have shown that constriction may be caused by noradrenalin, 5-hydroxytryptamine (5-HT, serotonin), acetylcholine, and angiotensin II, these substances have not appeared to be major cerebrospinal fluid (CSF)-borne mediators of prolonged cerebral arterial vasoconstriction in our model.

Recently, attention has been focused upon hemoglobin (Hb) as a possible cause of spasm. The oxidized form, oxyhemoglobin (oxyHb), is a known constrictor of cerebral vessels in vitro, and the time course of its appearance in the CSF after aneurysm rupture closely follows that of delayed cerebral vasospasm. Accordingly, it was of interest to study the effects on primates of intracisternal administration of pure oxyHb and methemoglobin (metHb). Particular attention was paid to the time course of any resultant arterial narrowing, the in vitro reactivity of the major cerebral arteries (at postmortem examination), their pathology, and the pharmacological vasoconstrictor activity of the CSF.

We now report that intracisternal administration of oxyHb causes cerebral vasoconstriction in primates, but the duration of spasm is shorter than in the clinical situation, and the pathological changes associated with human vessels after prolonged spasm (post-SAH) are not observed.

Materials and Methods

Preparation of Hemoglobin

Venous blood from normal male volunteers was drawn into (tri-) sodium citrate and carefully centrifuged to separate erythrocytes from platelet-rich plasma.
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**FIG. 1.** The absorption spectra of oxyhemoglobin (dotted line) and methemoglobin (solid line) used in this study. The ordinate shows the ultraviolet absorption of solutions of oxyhemoglobin and methemoglobin (0.5 g/ml H₂O); path length is 1 cm, and absorption is in arbitrary units. The abscissa shows the wavelength of incidental light (nm).

(PRP). The PRP was carefully removed, and the red cells washed five times with an equal volume of ice-cold 0.9% sodium chloride solution. The red cells were lysed with two volumes of ice-cold distilled water and shaken for 15 minutes. This solution was centrifuged at 100,000 G for 30 minutes to sediment the membrane fractions. All centrifugations were performed at 4°C. Oxyhemoglobin was then isolated by Sephadex G100 gel filtration. Small molecular weight contaminants were removed by Sephadex G25 gel filtration, and the resultant solution concentrated and de-salted by ultrafiltration with Amicon Centriflo filter cones. The concentrate, in Hartmann's solution (sodium lactate), was shown to be present, largely as oxyHB, by ultraviolet spectrophotometry (Fig. 1), and was found to migrate as a single band on SDS gel electrophoresis. A solution of human Type IV hemoglobin† in Hartmann's solution gave an ultraviolet absorbance spectrum characteristic of metHB.

**Dosage of Hemoglobin**

Little information is available on the amount of blood extravasated into the subarachnoid space during aneurysm rupture. Tourtellotte, et al., reported values as high as 1.6 × 10⁹ red cells/ml of lumbar CSF collected from SAH patients. Assuming a red cell count of 5.4 × 10¹¹/ml in blood from adult males and a CSF volume of 150 ml, then up to 44 ml of blood may be released into the CSF by the rupture of an intracranial aneurysm. This volume of blood is equivalent to 100 mg/kg of pure Hb in man. Accordingly, 53.3 to 58.6 mg/kg of oxyHb (the oxidized form of Hb) dissolved in Hartmann's solution was injected intracisternally into three baboons, and 50 mg/kg of metHb (the reduced form of Hb) injected into one baboon. Control experiments included a cisternal puncture but no injection, and an intracisternal injection of autologous CSF.

**Angiography and Arterial Measurement**

Baboons weighing 8 to 11 kg were anesthetized with 0.5% halothane in oxygen after tranquilization with phencyclidine, 5 to 10 mg/kg. Anesthesia was maintained as described previously, and angiography was performed on the day of Hb administration (Day 0) and then on Days 2, 3, and 7. Arterial diameters were measured from the angiograms to the nearest 0.1 mm and compared to control angiograms performed immediately before injection of Hb. The following arteries were measured: the supraclinoid and the terminal segments of the internal carotid artery (C₂ and C₃), and the proximal segments of the middle cerebral (MC₁) and the anterior cerebral arteries (AC₁). The diameters of the branches of the middle cerebral (MC₂, MC₃) and the anterior cerebral arteries (AC₃, AC₄) were also determined.

**Neurological Assessment**

The animals were observed throughout the period of the study, and continually assessed for motor neurological deficits and alterations in state of consciousness and behavior.

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*Amicon CF50A Centriflo filter cones manufactured by Amicon Corp., Lexington, Massachusetts.
† Human Type IV hemoglobin obtained from Sigma Chemical Co., Poole, Dorset, England.
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FIG. 3. The effect of a control intracisternal (I.C.) injection of autologous cerebrospinal fluid (CSF) on the caliber of baboon cerebral arteries. The change in arterial diameter is expressed as a percentage of the control diameter. Arrow denotes the intracisternal injection of 2 ml of autologous CSF.

Collection of Cerebrospinal Fluid

The animals were sedated with phencyclidine (5 to 10 mg/kg) on Day 8, and CSF sampled by cisternal puncture. The first droplet of CSF, possibly contaminated with blood from the passage of the needle through the tissues, was discarded. The remainder of the sample was collected in aliquots, so that overtly bloody CSF (which usually appeared at the end of CSF withdrawal) was not mixed with the bulk of the sample. The CSF was immediately cooled in ice. It was then centrifuged at 6500 G for 5 minutes in an Eppendorf bench centrifuge to remove whole cells and debris, assayed for Hb by a spectrophotometric method, and then frozen to -20°C.

Isolated Tissues

Animals were sacrificed with Euthanyl (sodium pentobarbitone, 20 to 40 mg/kg) on Day 8, and the brains removed. The basilar and vertebral arteries and those vessels comprising the anterior part of the circle of Willis were carefully dissected out and placed into ice-cold oxygenated Krebs solution (mmol/liter: NaCl 118.2; KCl 4.7; CaCl2·2H2O 2.52; MgSO4·7H2O 1.18; KH2PO4 1.18; NaHCO3 25.0; glucose 11.1) at a pH of 7.4. Within 12 to 15 hours, these were dissected into spiral strips and mounted in 5-ml organ baths in Krebs solution maintained at 37°C and continuously oxygenated with a mixture of 95% O2 and 5% CO2.

Vasoconstriction was measured isotonically with a Harvard 386 transducer attached to a potentiometric chart recorder.‡ We measured vasoconstrictor responses to serotonin (in a 2 to 2000 nmol/liter bath concentration), and oxyHb and metHb (in 38 to 50 mg/ml bath concentrations, mimicking those expected in the CSF immediately after Hb injection). Arteries were also challenged with autologous CSF and vasoconstrictor responses of each tissue were quantitated in terms of the bath concentration of serotonin (nmol/liter) required to elicit a contraction of the same magnitude as the CSF response. These values were derived from dose-response curves for 5-HT obtained for individual preparations to allow comparison of response between preparations of varying sensitivity.

Neuropathological Assessment

On removal of the brain immediately after sacrifice, segments of the basilar and vertebral arteries and the circle of Willis were fixed with 10% formalin solution for subsequent staining and microscopic examination. The brains were similarly preserved, sectioned, and examined for evidence of infarction and other histological changes.10

Results

Angiography

Cisternal puncture followed by repeated cerebral angiography had no effect on the caliber of baboon cerebral arteries (Fig. 2). A cisternal puncture with injection of autologous CSF produced a similar lack of response (Fig. 3). In a single experiment, intracisternal metHb failed to produce prolonged angiographically determined cerebral arterial narrowing, and slight dilation of the vessels was seen 7 days after injection (Table 1 and Fig. 4). In three further experiments, intracisternal injection of oxyHb produced rapidly developing spasm. Arterial constriction was evident 5 minutes after injection; spasm was maximal after 1 hour, and persisted for 2 days. However, on Day 3, all vessels showed slight vasodilatation. Seven days after injection, slight spasm

‡ Chart recorder manufactured by Tekman Co., Bicester, England.
TABLE 1
Effect of autologous CSF and vasospastic CSF on isolated baboon intracranial arteries

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Pretreatment Agent</th>
<th>Arterial Caliber† (% change)</th>
<th>Contractile Response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>To Autologous CSF</td>
<td>To Vasospastic CSF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Response</td>
<td>No.</td>
</tr>
<tr>
<td>1</td>
<td>metHb</td>
<td>100.5 ± 5.06</td>
<td>0.15 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>oxyHb</td>
<td>114.7 ± 3.28</td>
<td>3.30 ± 0.95</td>
</tr>
<tr>
<td>3</td>
<td>oxyHb</td>
<td>76.8 ± 2.30</td>
<td>5.40 ± 2.22</td>
</tr>
<tr>
<td>4</td>
<td>oxyHb</td>
<td>90.0 ± 4.02</td>
<td>1.92 ± 1.92</td>
</tr>
<tr>
<td>mean values (animals 2-4)</td>
<td>90.6 ± 10.7</td>
<td>3.54 ± 1.01</td>
<td>10.9 ± 3.72</td>
</tr>
</tbody>
</table>

* Animals were pretreated 8 days before cerebrospinal fluid (CSF) sampling with either oxyhemoglobin (oxyHb) or methemoglobin (metHb) (50.0 to 58.8 mg/kg) administered intracisternally.
† Mean arterial change ± SE, determined from angiograms on the day of sampling 8 days after pretreatment and expressed as a percentage of the mean control arterial caliber.
‡ Isolated intracranial arteries obtained immediately after sacrifice were challenged with autologous CSF drawn at the time of death, and with CSF obtained from a patient after SAH due to aneurysm rupture. The CSF (500 μl) was added to a 5-ml organ bath. Contractile responses are given as mean responses ± SE (where applicable) in terms of 5-HT vasoconstrictor equivalents (nmol/liter bath concentration). No. denotes the number of isolated baboon cerebral artery preparations challenged with each CSF sample.

had returned (Table 1 and Figs. 4 and 5). The vasoconstrictor effects were most pronounced on the proximal portions of the intracranial arteries (C2, C3, MC1, AC1), as shown in Fig. 6.

Isolated Tissues

Samples of CSF taken on Day 8 from each baboon contained no detectable Hb. The lower limit of detection for Hb was 0.64 mg/ml. Intracranial arteries (basilar, vertebral, and circle of Willis) removed immediately after death on Day 8 all showed dose-related vasoconstrictor responses to 5-HT in vitro. Contractile responses to autologous CSF were, however, markedly smaller (mean response ± SE: 0.15 ± 0.15 nmol/liter serotonin equivalents in four preparations) for vessels from the single animal treated with metHb than those for vessels from the three animals after oxyHb (3.54 ± 1.01 nmol/liter 5-HT equivalents in 13 preparations) as shown in Table 1. In contrast, arteries from the baboon treated with metHb showed greater responsiveness (21.9 nmol/liter 5-HT equivalents in two preparations) to a sample of vasospastic CSF from a patient after SAH than did arteries from the animals treated with oxyHb (10.9 ± 3.7 nmol/liter 5-HT equivalents in seven preparations). Table 1 also demonstrates that the largest vasoconstrictor response to autologous CSF (5.40 ± 2.22 nmol/liter 5-HT equivalents in four preparations) was seen with vessels from the animal showing the greatest arterial narrowing (76.8 ± 2.30% of control diameter) on Day 7 after oxyHb injection but the smallest response to human vasospastic CSF (mean response 6.8 nmol/liter 5-HT equivalents in two preparations). Isolated intracranial arteries from all animals contracted to metHb or oxyHb in bath concentrations of 38 to 50 mg/ml.

Neuropathology

Microscopic examination of the intracranial arteries from each animal showed none of the signs (such as subintimal thickening with intimal necrosis) characteristic of a vessel after a period of prolonged spasm induced by subarachnoid blood. Brain slices did not show conclusive evidence of infarction. None of the experimental animals demonstrated neurological deficits attributable to focal cerebral ischemia.

Discussion

Of many substances postulated to be involved in the etiology of prolonged cerebral vasospasm after aneurysm rupture, hemoglobin (Hb) has, perhaps, most claim to a major role. It is a relatively potent contractile agent in isolated canine intracranial artery preparations. Its in vitro activity is not mediated by histaminergic, serotoninergic, or adrenergic receptors. In this respect, its action is similar to the vasoactivity seen in...
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CSF obtained from SAH patients after prolonged cerebral vasospasm. Furthermore, the appearance of Hb in the CSF after SAH closely follows the documented time course of spasm, obviating the necessity for it to possess prolonged in vitro vasoconstrictor activity in order to reproduce the sustained cerebral arterial narrowing characteristic of the syndrome.

Little in vivo investigation of the effects of Hb has been undertaken. Ishii and Nonaka administered oxyHb intracisternally to 22 dogs, and observed angiographic arterial narrowing at 3 and 7 days after Hb injection in 11 and 16 animals, respectively. The data we have presented demonstrate that oxyHb was capable of causing arterial narrowing that lasted for 3 days in an established primate model of cerebral arterial spasm. However, prolonged arterial narrowing of the severity of that seen with simulated aneurysm rupture or intracisternal injection of blood was not observed. Neither were histopathological arterial changes characteristic of prolonged spasm nor gross signs of related ischemic lesions in evidence. It would, therefore, seem that oxyHb alone, administered intracisternally without simulated aneurysm rupture, is not capable of inducing the cerebral vasospasm syndrome during life in baboons with undamaged arteries.

The observed vasoconstrictor effect of Hb appeared to be dependent on the oxidation state of the compound. In a single experiment, metHb, the reduced form of oxyHb, did not cause significant reduction in cerebral arterial diameter. This confirms reports that metHb lacks in vitro vasoconstrictor activity. The presence of a highly oxidized compound in the subarachnoid space also affected the vasoactivity detected in the CSF at sacrifice. No Hb was detected in the CSF, but vasoconstrictor activity was found to be considerably greater in CSF from animals after intracisternal administration of oxyHb. This finding was not thought to be an artifact of diminished arterial sensitivity to CSF-borne vasoconstrictors caused by intracisternal injection of metHb. Vessels from the animal treated with metHb showed the largest responses to a sample of human CSF obtained from a patient with angiographic spasm after aneurysm rupture. Interestingly, the greatest vasoconstrictor activity was detected in CSF from the animal with the greatest arterial narrowing at sacrifice (Table 1), implying a link between angiographic spasm and the pharmacological activity of the CSF.

The presence of vasoconstrictor material in the CSF, in the absence of detectable Hb concentrations, supports the proposition of Sano, et al., that stimulation of lipid peroxidation in the subarachnoid CSF by oxidized Hb might result in the generation of vasoactive lipid hydroperoxides. However, the results of the present study indicate that such an effect of intracisternal administration of oxyHb, even combined with its direct vasoconstrictor actions, is insufficient to produce the prolonged arterial narrowing and histological changes associated with the cerebral vasospasm syndrome.

References


Manuscript received November 23, 1982. Accepted in final form February 2, 1983.

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