Hemoglobin released from hemolysed erythrocytes has been postulated to be responsible for delayed cerebral vasospasm after subarachnoid hemorrhage (SAH). However, the evidence is indirect because it is based on the measurement of hemoglobin, or its products, in cerebrospinal fluid (CSF) that was collected at a distance from the artery in spasm. In addition, because of its impurity, the use of bovine hemoglobin in some in vivo experiments makes the results inconclusive. Furthermore, recent magnetic resonance imaging studies have shown that after intracranial hemorrhage oxyhemoglobin disappears quickly from the intracranial clot, which suggests that deoxyhemoglobin, not oxyhemoglobin, may be responsible for evoking vasospasm. We examined the role of hemoglobin in the development of vasospasm by using an in vivo “closed” microdialysis system that allowed measurement of time-dependent changes of oxyhemoglobin, deoxyhemoglobin, and methemoglobin in the perivascular space of a primate model of SAH.

Materials and Methods

In vivo Microdialysis System

Nine cynomolgus monkeys received intramuscular injections of atropine sulfate (0.05 mg/kg), sodium thiopental (25 mg/kg), dexamethasone (0.7 mg/kg), cefazolin (500 mg), and ketamine (10 mg/kg) preoperatively. They underwent intubation and ventilation with N2O/O2 (1:1), and 0.5% to 1% isoflurane was used as the anesthetic. The expired PCO2 level was maintained at 40 mm Hg by ventilatory control and confirmed by measurement of arterial blood gas levels. A right frontotemporal craniectomy was performed under aseptic conditions, and the arachnoid over the proximal portion of the middle cerebral artery (MCA) and the bifurcation of the internal carotid artery was opened by sharp dissection. A microdialysis catheter adjacent to the right middle cerebral artery (MCA). Saline (control group, three animals) or an arterial blood clot (SAH group, six animals) was then placed around the MCA and the catheter. Arteriographically confirmed vasospasm had developed in all animals with SAH but in none of the control animals on Day 7. The dialysate was collected daily for 12 days. Levels of oxyhemoglobin, deoxyhemoglobin, and methemoglobin were measured by means of spectrophotometry.

Perivascular concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin peaked on Day 2 in the control monkeys and could not be detected on Days 5 to 12. Perivascular concentrations of oxyhemoglobin and deoxyhemoglobin peaked on Day 7 in the SAH group, at which time the concentrations in the dialysate were 100-fold higher than in any sample obtained from the control animals. Methemoglobin levels increased only slightly, peaking between Days 7 and 12, at which time the concentration in the dialysate was 10-fold higher than in samples from the control animals.

Conclusions. This study provides in vivo evidence that the concentrations of oxyhemoglobin and deoxyhemoglobin increase in the cerebral subarachnoid perivascular space during the development of delayed cerebral vasospasm. The results support the hypothesis that oxyhemoglobin is involved in the pathogenesis of delayed cerebral vasospasm after SAH and implicated deoxyhemoglobin as a possible vasoconstrictive agent.

Key Words • cerebral vasospasm • oxyhemoglobin • deoxyhemoglobin • methemoglobin • microdialysis • subarachnoid hemorrhage • Macaca cynomolgus
catheter made from a semipermeable membrane with a 100-KD molecular weight cut-off was placed above the right MCA. The artery and catheter were then covered with 5 ml of saline (control group, three monkeys) or preclotted arterial blood (SAH group, six monkeys).1,2,3,4 One end of the catheter was connected to a microcros-
motic pump and the other end was connected to an Omnarray reservoir (volume 0.54 ml) buried under the skin on the animal’s back. The total volume of fluid inside the catheter and the microdialysis fiber was 0.03 to 0.04 ml. The pump delivered 0.28 ml of distilled water per day for 7 days. It was changed on Day 7 when the animal was anesthetized for the postoperative arteriography. A preliminary in vitro study revealed that measured concentrations of hemoglobin were 70 ± 8% of the available hemoglobin (10 ^-3 M; measured three times) using this microdialysis system. The animals were killed on Day 13 post-surgery, and the position of the catheter was confirmed at autopsy.

Arteriographic Studies

To assess vasospasm, cerebral arteriography was performed 4 days before surgery and on Day 7 postoperatively, using methods described elsewhere.4 Briefly, the monkeys were anesthetized with intramuscular injections of ketamine (10 mg/kg) and xylazine (Rompun, 1 mg/kg) and intubated and ventilated (PCO2 = 40 mm Hg). A femoral artery cutdown was performed under aseptic condi-
tions, and a No. 3 (animals weighing < 5 kg) or No. 4 (animals weighing > 5 kg) French polyethylene catheter was advanced with the aid of fluoroscopic guidance to the right internal carotid artery. Contrast medium (0.5–0.75 ml Conray 60%) was injected by hand. The filming sequence was conducted at two films per second for 3 seconds, and then one film per second for 6 seconds. All filming was performed at a magnification factor of 2. Subtraction films of the anteroposterior (AP) projections were made. The presence of vasospasm was detected by comparing the results of cerebral ar-
teriographic studies of the right MCA obtained before surgery with the results of studies obtained on Day 7 post-surgery. A computer-
ized image analysis system for the Macintosh computer was used to measure the area of the proximal 14 mm of the right MCA using the AP projection. Vasospasm was recognized if the pre- and postoper-
ative arteriographic measurements of the right MCA indicated nar-
rrowing of the vessel lumen (11–25% reduction in AP area, mild vasospasm; 26–50%, moderate vasospasm; and > 50%, severe va-
sospasm).

Sample Collection

Microdialysate samples, approximately 0.28 ml each day, were collected by direct puncture of the Omnarray reservoir daily for 12 days and were stored at −70°C. Because the pump was primed before placement under the skin and there was a “dead space” in the catheters (0.03–0.04 ml), samples obtained on the 1st day post-
surgery were not used for analysis.

Measurements of Hemoglobin

The concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin were measured by means of spectrophotometry. The absorbance of the samples was measured three times at each wavelength (A560, A576, A578, and A630) and the mean value of the measurements was used for calculation of the hemoglobin concentrations as follows: for oxyhemoglobin:2,3 [Oxy Hb] = (1.55 A578 − 0.861 A562 − 0.689 A598) / 6.45; for deoxyhemoglobin:1 [Deoxy Hb] = (1.373 A560 − 0.747 A576 − 0.737 A630) × 10 ^-4; for methemoglobin:1 [Met Hb] = (2.985 A630 + 0.194 A576 − 0.4023 A560) × 10 ^-4. The values are presented as 10 ^-4 M ± the standard error of the mean.

Statistical Analysis

The data were analyzed by analysis of variance, with data from postoperative Days 2 through 12 used for the calculations. The values for Day 1 were excluded from the analysis because the oxyhe-
moglobin concentrations were exceptionally high, probably because of extracorporeal preparation of the clot. This theory was confirmed by an in vitro study that showed an increase of oxyhemoglobin in mock CSF immediately after exposure to the extracorporeally clotted arterial blood. Statistical significance was defined as a probab-
ility value of less than 0.05.

Sources of Supplies and Equipment

The microdialysis catheters were obtained from CMA/Micro-
dialysis, Dalvagen, Sweden, and the microcromotic pump (Alzet 2ML1) was purchased from Alza, Inc., Palo Alto, CA. The contrast medium was acquired from Mallinckrodt Medical, Inc., St. Louis, MO. The image analysis system software (Image 1.54) was developed by Wayne Rasband, National Institutes of Mental Health, Bethesda, MD, The Macintosh computer was purchased from Apple Computer Corp., Cupertino, CA, and the Statview+Graphics soft-
ware was supplied by Abacus Concepts, Inc., Berkeley, CA.

Results

Arteriographic vasospasm of the right MCA developed in all monkeys with subarachnoid clots (11–50% decrease of the projected AP area of the right MCA; 32 ± 13%; mean ± standard deviation) but in none of the control monkeys (−9 to 9% decrease; −2 ± 1%). In the control monkeys the hemoglobin levels peaked on Day 2 (oxyhe-
moglobin, 8 ± 6 × 10 ^-7 M; deoxyhemoglobin 3 ± 5 × 10 ^-7 M; methemoglobin 2 ± 3 × 10 ^-4 M; p < 0.05 com-
pared with Days 5–12) and then disappeared from the micro-
dialysate samples on Day 5. In the monkeys with SAH the concentration of hemoglobins was much higher than in the control animals. The oxyhemoglobin levels (Fig. 1A) started to increase on Day 5 and peaked (0.5 ± 0.2 × 10 ^-4 M) on Day 7 after clot placement (p < 0.05 com-
pared with Days 3, 4, 5, 7, 8, 9, 11, and 12). The deoxyhe-
moglobin levels (Fig. 1B) also increased (0.2 ± 0.2 × 10 ^-4 M) on Day 7 after clot placement (p < 0.05 compared with Days 3–5, and 8). The methemoglobin levels (Fig. 1C) did not change appreciably between Days 2 and 11 but peaked on Day 12 (p < 0.05 compared with Days 2, 4, 5, and 7).

Discussion

Microdialysis System

Ever since delayed cerebral vasospasm after SAH was related to posthemorrhagic changes in CSF,5 research on the etiology of vasospasm has focused on blood, erythro-
cytes, and hemoglobin6,9,30 and its metabolites.8,10,14,15,17,20,25,29,30

Oxyhemoglobin, a product of erythrocyte lysis, seems to be the molecule that is most likely to be responsible for the occurrence of delayed vasospasm after SAH,29 although methemoglobin,6,30 bilirubin,7,32,40 hemin,25 and iron ions30,32,38 have also been implicated. The major difficulty with these various studies is that the concentrations of the different substances in the CSF samples collected from the ventricles, lumbar space, or subarachnoid cisterns are extrapolated to be the concentrations in the perivascular space immediately contiguous to spastic vessels. It is known, however, that the concentrations of CSF constitu-
ents differ depending on the region from which they were collected.24 Moreover, the concentrations of CSF constitu-
ents are different in the perivascular space and the sub-
arachnoid space.52 Another pitfall in previous experiments is directly related to the measurement of oxyhemoglobin in the CSF, because deoxyhemoglobin in the CSF is con-
verted to oxyhemoglobin34 by exposure to the oxygen in the fluid,34 as well as by exposure of the CSF samples to air. The oxyhemoglobin levels in the CSF samples could, therefore, be artificially inflated. These problems may un-
derlie the wide variation in the correlation between the CSF oxyhemoglobin concentrations and cerebral vasospasm. In an attempt to overcome these problems, we developed an in vivo closed microdialysis system for continuous monitoring of hemoglobin in the perivascular space. Because the molecular weight of hemoglobin is 64,500, we used a semipermeable membrane with a very high molecular weight cut-off (100 kD). This system allowed us to collect fluid samples from the space directly surrounding the vessel. We monitored changes in the concentrations of the hemoglobins in the direct vicinity of a vessel during the development of posthemorrhagic cerebral vasospasm. This method, however, does not measure the exact levels of hemoglobin in the perivascular space; the levels of hemoglobin measured in in vitro testing were 70 ± 8% of the known hemoglobin concentration in the solution. Furthermore, the partial removal of hemoglobin from the perivascular space by sample collection did not affect the incidence or degree of vasospasm characteristic of this model. 

Levels of Hemoglobins

Oxyhemoglobin. Although oxyhemoglobin has long been considered responsible for evoking vasospasm, the experimental and clinical evidence supporting this hypothesis is indirect. The reported levels of hemoglobin range from $5 \times 10^{-4}$ M in a subarachnoid hematoma to $3 \times 10^{-5}$ M in the CSF. In the current study microdialysis detected corresponding levels of oxyhemoglobin ($0.5 \times 10^{-4}$ M). However, the true level of hemoglobin could be approximately 30% higher than the values measured here, for the reason mentioned previously. Nevertheless, the results demonstrate the usefulness of our method for assessing the perivascular concentration of hemoglobins and support a role for oxyhemoglobin in the development of delayed cerebral vasospasm after SAH. The results also indicate that deoxyhemoglobin may contribute to the development of vasospasm.

Deoxyhemoglobin. This hemoglobin has received scant attention in delayed cerebral vasospasm, perhaps because investigational samples are usually collected from the CSF, in which oxygen tension is high (31–47 mm Hg) and in which at least 75% of deoxyhemoglobin is converted to oxyhemoglobin. Exposure of test samples to oxygen levels that are higher than those within the clot could falsely produce the impression that oxyhemoglobin, and not deoxyhemoglobin, is present in the CSF samples. Because our method of measuring hemoglobin levels (spectrophotometry) did not allow us to continue anaerobic conditions, we also could not completely avoid errors of oxygen exposure. Nevertheless, despite the conversion of deoxyhemoglobin to oxyhemoglobin during measurement, a significant increase in deoxyhemoglobin occurred in the perivascular samples at the time of vasospasm, and the level of deoxyhemoglobin was significantly higher change appreciably between Days 2 and 11; however, they peaked on Day 12. *p < 0.05 compared with the levels on Days 2, 4, 5, and 7. In three sham-operated control monkeys levels of oxyhemoglobin, deoxyhemoglobin, and methemoglobin never exceeded $2 \times 10^{-6}$ M between Days 2 and 4 and were below the level of detection on Days 5 to 12.
than earlier values reported in the CSF (0.003–5 × 10⁻⁵ M compared with 0.2 ± 0.2 × 10⁻⁴ M).

Potential involvement of deoxyhemoglobin in the production of vasospasm after SAH is further supported by a recent magnetic resonance imaging study in which oxyhemoglobin is rapidly (within hours) and completely converted into deoxyhemoglobin after hemorrhage, especially in the thick layer of subarachnoid blood that is known to be responsible for delayed vasospasm.

Methemoglobin. Levels of this hemoglobin did not change significantly during the time of vasospasm but significantly increased on Day 12, when vasospasm in this model usually resolves. Thus, these results confirm that methemoglobin is not directly involved in vasospasm, as suggested earlier.

Pathophysiological Mechanisms of Vasospasm

Although oxyhemoglobin has been widely accepted as a direct cause of vasospasm, it is still unclear which of its diverse mechanisms of action, if any, is responsible for evoking vasospasm. Recent studies have excluded or supported the following mechanisms: formation of oxygen radicals, release of free radicals and initiation of lipid peroxidation, release of eicosanoids, development of proliferative arteriopathy, and release of endothelin-1.

Other mechanisms, such as scavenging of endothelium-derived relaxing factor (EDRF)–nitric oxide (NO) damage to adventitial nerves remain under investigation.

Our results confirm a role for oxyhemoglobin and/or deoxyhemoglobin as the cause of vasospasm after SAH by the correlation of peak levels of perivascular oxyhemoglobin and deoxyhemoglobin during the interval of delayed cerebral vasospasm. Recognition of NO as an EDRF and the description of a "sink effect" in which hemoglobin binds NO explain a potential mechanism by which oxyhemoglobin decreases EDRF.

This mechanism is further supported by the reversal of vasospasm by a direct intracarotid infusion of an NO donor. However, deoxyhemoglobin, as oxyhemoglobin, also scavenges NO in a reaction that produces a stable nitrosylhemoglobin. Thus, both hemoglobins eliminate the action of this vasodilatory agent and vasospasm could be produced by the unopposed action of constricting agents like endothelin.

The existence of such a mechanism is further supported by the loss of vasodilator properties of the vessel wall with preservation of its vasoconstrictive abilities. Although the limited penetration of oxyhemoglobin in the vessel wall may preclude a dramatic decrease in the availability of the NO that is produced by endothelial cells, it has been shown that NO synthase (NOS) activity is present in the nerve endings in the adventitia of conductive cerebral vessels as another source of NO for the vessel wall.

An arterial nerve plexus is formed by the nerves from the sphenopalatine ganglion, stimulation of which increases CBF by vasodilation of the cerebral vessels. Earlier reports have shown the disappearance of the nerve endings from the adventitia of vessels exposed to blood oxygenated-induced damage to the NOS-containing vasodilatory nerves. This hypothesis of the involvement of adventitial NOS-positive neurons in the production of vasospasm has been recently confirmed by our laboratory, demonstrating the disappearance of NOS activity from the adventitial nerve endings in the cerebral vessels in spasm, when it is at the same time immunoreactivity of endothelial NOS was preserved in these vessels, which suggests a loss of vasodilatory input from the adventitia in response to the presence of blood in the subarachnoid space, and not a loss of NO produced by endothelial cells, as the cause of vasospasm.

The current experiment provides further evidence for direct involvement of oxyhemoglobin and deoxyhemoglobin, but not methemoglobin, in the development of delayed cerebral vasospasm. Deoxyhemoglobin involvement in vasospasm is further supported by our recent observation that treatment with a ferrous but not a ferric iron chelator prevents development of vasospasm in the primate model of SAH. The influence of deoxyhemoglobin on NOS activity in the adventitia and scavenging of NO needs further elucidation.

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