Source and cause of endothelin-1 release into cerebrospinal fluid after subarachnoid hemorrhage

Ryszard M. Pluta, M.D., Ph.D., Robert J. Boock, Ph.D., John K. Afshar, M.D., Kathleen Clouse, Ph.D., Mima Bacic, M.D., Ph.D., Hannelore Ehrenreich, M.D., Ph.D., and Edward H. Oldfield, M.D.

Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland; Center for Biologics Evaluation and Research, United States Food and Drug Administration, Bethesda, Maryland; and Department of Neurology and Psychiatry, University of Göttingen, Göttingen, Germany

Despite years of research, delayed cerebral vasospasm remains a serious complication of subarachnoid hemorrhage (SAH). Recently, it has been proposed that endothelin-1 (ET-1) mediates vasospasm. The authors examined this hypothesis in a series of experiments. In a primate model of SAH, serial ET-1 levels were measured in samples from the perivascular space by using a microdialysis technique and in cerebrospinal fluid (CSF) and plasma during the development and resolution of delayed vasospasm. To determine whether elevated ET-1 production was a direct cause of vasospasm or acted secondary to ischemia, the authors also measured ET-1 levels in plasma and CSF after transient cerebral ischemia. To elucidate the source of ET-1, they measured its production in cultures of endothelial cells and astrocytes exposed to oxyhemoglobin (10 μM), methemoglobin (10 μM), or hypoxia (11% oxygen). There was no correlation between the perivascular levels of ET-1 and the development of vasospasm or its resolution. Cerebrospinal fluid and plasma levels of ET-1 were not affected by vasospasm (CSF ET-1 levels were 9.3 ± 2.2 pg/ml and ET-1 plasma levels were 1.2 ± 0.6 pg/ml) before SAH and remained unchanged when vasospasm developed (7.1 ± 1.7 pg/ml in CSF and 2.7 ± 1.5 pg/ml in plasma). Transient cerebral ischemia evoked an increase of ET-1 levels in CSF (1 ± 0.4 pg/ml at the occlusion vs. 3.1 ± 0.6 pg/ml 4 hours after reperfusion; p < 0.05), which returned to normal (0.7 ± 0.3 pg/ml) after 24 hours. Endothelial cells and astrocytes in culture showed inhibition of ET-1 production 6 hours after exposure to hemoglobins. Hypoxia inhibited ET-1 release by endothelial cells at 24 hours (6.4 ± 0.8 pg/ml vs. 0.1 ± 0.1 pg/ml, control vs. hypoxic endothelial cells; p < 0.05) and at 48 hours (6.4 ± 0.6 pg/ml vs. 0 ± 0.1 pg/ml, control vs. hypoxic endothelial cells; p < 0.05), but in astrocytes hypoxia induced an increase of ET-1 at 6 hours (1.5 ± 0.6 vs. 6.4 ± 1.1 pg/ml, control vs. hypoxic astrocytes; p < 0.05).

Endothelin-1 is released from astrocytes, but not endothelial cells, during hypoxia and is released from the brain after transient ischemia. There is no relationship between ET-1 and vasospasm or oxyhemoglobin, a putative agent of vasospasm, in vitro. The increase in ET-1 levels in CSF after SAH from a ruptured intracranial aneurysm appears to be the result of cerebral ischemia rather than reflecting the cause of cerebral vasospasm.

KEY WORDS • endothelin • vasospasm • subarachnoid hemorrhage • cerebral ischemia • cynomolgus monkey

Oxyhemoglobin is a putative agent of delayed cerebral vasospasm after subarachnoid hemorrhage (SAH). One of the potential oxyhemoglobin-related mechanisms of vasospasm is the release of endothelin-1 (ET-1), a potent endothelium-derived vasoconstricting agent. Not only are astrocytes, neurons, and pituitary cells a normal source of extraluminal ET-1, but endothelial cells and smooth-muscle cells also release ET-1 when stimulated by oxyhemoglobin or thrombin. Because an increase in the levels of ET-1 in CSF has been described in patients with cerebral vasospasm and delayed ischemic neurological deficits after SAH, the production of ET-1 may also be induced by ischemia. Thus, not only is the source of ET-1 undefined, but the mechanism of ET-1 release is unclear after SAH. To clarify these issues, we conducted a series of in vivo and in vitro experiments to answer the following questions: Is ET-1 secreted into the perivascular space during cerebral vasospasm? Is there a relationship between plasma or CSF levels of ET-1 and vasospasm? Does oxyhemoglobin induce the release of ET-1 from astrocytes or endothelial cells? Is hypoxia–ischemia responsible for ET-1 release from cell cultures and the brain?

Materials and Methods

In Vivo Studies

Animal Preparation. Cynomolgus monkeys were used for all in vivo studies. The protocols were reviewed by the National Institute of Neurological Disorders and Stroke Animal Care and Use
Committee and met the National Institutes of Health guidelines for animal care. Before surgery the monkeys received atropine sulfate (0.05 mg/kg), sodium thiopental (25 mg/kg), and ketamine (10 mg/kg). They were intubated and ventilated with O2/O (1:1) and anesthetized with 0.5% isoflurane. The expired PaCO2 level was maintained at 40 mm Hg by ventilatory control and confirmed by measurement of arterial blood gas levels.

**Vasospasm and ET-1 in the Perivascular Space.** Six monkeys underwent right-sided frontotemporal craniotomy under aseptic conditions. The arachnoid over the proximal portion of the middle cerebral artery (MCA) and the bifurcation of the internal carotid artery (ICA) were sharply opened. A semipermeable catheter, constructed using a membrane with a 100-kD molecular-weight cutoff, was placed around the right MCA. The artery and catheter were covered with 5 ml of precollected arterial blood.7,28 One end of the catheter was connected to a microsomatic pump and the other end was connected to an Ommaya reservoir (side inlet 1.5 cm; volume 0.53 ml) buried under the scalp. The total volume of fluid inside the catheter and the microdialysis fiber was 0.03 to 0.04 ml. The pump constantly delivered 0.28 ml of distilled water each day for 7 days. It was changed on Day 7 when postoperative angiography was performed.

An in vitro pilot study revealed that 80% ± 5% of the available ET-1 (10 pg/ml) was recovered from the saline samples by using this microdialysis system.

The microdialysate samples, approximately 0.28 ml each, were collected by direct puncture of the Ommaya reservoir daily for 12 days. The samples were stored at −70°C. Because the pump was primed immediately before the samples and because of the existence of a “dead space” in the catheters (0.03–0.04 ml), the samples from postoperative Day 1 were not used for analysis. The animals were killed on postoperative Day 13 and the position of the catheter was confirmed at autopsy.

For assessment of vasospasm, cerebral angiography was performed on preoperative Day 4 and on postoperative Day 7, using methods described elsewhere.12,44 The monkeys were anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (Rompun, 1 mg/kg). A femoral artery cutdown was performed under aseptic conditions and a No. 3 (in animals weighing < 5 kg) or No. 4 (in animals weighing > 5 kg) French polyethylene catheter was advanced, under fluoroscopic guidance, to the right ICA. The contrast medium (0.5–0.75 ml Conray 60%) was injected by hand. The filming sequence was two films per second for 3 seconds followed by one film per second for 6 seconds. All filming was conducted at a magnification factor of 2. Subtraction films of the anteroposterior (AP) projections were made. For grading vasospasm, the areas of the proximal 14 mm of the right MCA on preoperative and postoperative AP angiograms were measured by means of a computerized image analysis system. Vasospasm was recognized if the pre- and postoperative angiographic measurements of the area of the right MCA in the AP view indicated narrowing of the vessel lumen (11–25% reduction in AP area, mild vasospasm; 26–50%, moderate vasospasm; and > 50%, severe vasospasm).31

**Vasospasm and ET-1 in Plasma and CSF.** Eight monkeys underwent right-sided frontotemporal craniectomy with dissection of the arachnoid of the sylvian fissure, as described earlier.12,44 After the monkeys were anesthetized, a clot of arterial blood was placed around the right MCA. The artery and catheter were covered with 5 ml of precollected arterial blood.7,28 One end of the catheter was attached to a microsomatic pump and the other end was connected to a small secondary chamber in which the cells were confluent (3.4 × 10^5 cells/well) and the microdialysis fiber was 0.03 to 0.04 ml. The pump constantly delivered 0.28 ml of distilled water each day for 7 days. It was changed on Day 7 when postoperative angiography was performed.

An in vitro pilot study revealed that 80% ± 5% of the available ET-1 (10 pg/ml) was recovered from the saline samples by using this microdialysis system.

The microdialysate samples, approximately 0.28 ml each, were collected by direct puncture of the Ommaya reservoir daily for 12 days. The samples were stored at −70°C. Because the pump was primed immediately before the samples and because of the existence of a “dead space” in the catheters (0.03–0.04 ml), the samples from postoperative Day 1 were not used for analysis. The animals were killed on postoperative Day 13 and the position of the catheter was confirmed at autopsy.

For assessment of vasospasm, cerebral angiography was performed on preoperative Day 4 and on postoperative Day 7, using methods described elsewhere.12,44 The monkeys were anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (Rompun, 1 mg/kg). A femoral artery cutdown was performed under aseptic conditions and a No. 3 (in animals weighing < 5 kg) or No. 4 (in animals weighing > 5 kg) French polyethylene catheter was advanced, under fluoroscopic guidance, to the right ICA. The contrast medium (0.5–0.75 ml Conray 60%) was injected by hand. The filming sequence was two films per second for 3 seconds followed by one film per second for 6 seconds. All filming was conducted at a magnification factor of 2. Subtraction films of the anteroposterior (AP) projections were made. For grading vasospasm, the areas of the proximal 14 mm of the right MCA on preoperative and postoperative AP angiograms were measured by means of a computerized image analysis system. Vasospasm was recognized if the pre- and postoperative angiographic measurements of the area of the right MCA in the AP view indicated narrowing of the vessel lumen (11–25% reduction in AP area, mild vasospasm; 26–50%, moderate vasospasm; and > 50%, severe vasospasm).31

**Vasospasm and ET-1 in Plasma and CSF.** Eight monkeys underwent right-sided frontotemporal craniectomy with dissection of the arachnoid of the sylvian fissure, as described earlier.12,44 After the right MCA was exposed, a clot of arterial blood was placed around the artery. Cerebral angiography was performed 2 days before the SAH occurred and 7 days after surgery. Cerebrospinal fluid samples were collected directly from the basal cisterns during the operation for clot placement and by direct puncture of the cisterna magna on postoperative Day 7, when vasospasm was confirmed by angiography. Plasma samples were collected at the same time. All samples were stored at −70°C.

**Cerebral Ischemia and ET-1 in Plasma and CSF.** After general anesthesia had been induced in the monkeys, partial global cerebral ischemia was produced in five of them by bilateral extracranial occlusion of the ICA and common carotid artery (CCA) with temporary vascular clips for 30 minutes. Three monkeys from the group with transient ischemia underwent a small right-sided parietal craniectomy. After the dura was opened, a cerebral blood flow (CBF) probe was slipped between the dura and the brain to lie over a region perfused by the right MCA. The position of the probe was confirmed by skull x-ray films. After calibration of the instrument, regional CBF was measured continuously before, during, and after transient ischemia.

For collection of CSF samples at intervals, the back of the monkey’s head was shaved and scrubbed. The suboccipital membrane was exposed. A thin (PE 10) catheter was introduced into the cisterna magna, and the hole in the dura was closed with a No. 7.0 nylon suture and sealed with tissue glue. The external end of the catheter was also closed with glue and reopened for collection of 1 ml of CSF before placement of the vascular clips (control), at the start of reperfusion, and 0.5, 1, 2, 3, 4, 5, 6, and 24 hours after reperfusion. Plasma samples were collected concurrently. After 6 hours the external end of the catheter was sealed again, buried under the skin, and the anesthesia was reversed. Animals with neurological deficits were killed. Two monkeys without deficits were returned to their cages and anesthetized for collection of CSF samples again the next day (24 hours after reperfusion). The intracisternal catheter was exposed, the samples of CSF and plasma were collected, and the remaining animals were killed.

**In Vivo Studies**

**Cell Cultures.** Endothelial cells were isolated from human brain microvessels obtained from the resected temporal cortex in patients with epilepsy. Cell lines were established and maintained using human brain endothelium-specific medium consisting of Dulbecco’s modified Eagle medium (DMEM) and 35 ml of Media 199, as previously described.16 Endothelial cell lines were confirmed by Factor VIII-related antigen staining, using methods described elsewhere.24 The cells were confluent (1.3 ± 0.59 × 10^5 cells/well), and cells between passages 4 and 7 were used. The cells were exposed to various agents or environmental stimuli. The number of viable cells was assessed after each experiment: the cells were tripanned and then counted.

Astrocytes were collected from 2-week-old rat fetuses and a primary culture was established. Cell lines were maintained using DC-10 medium consisting of 500 ml of DMEM, 50 ml of fetal bovine serum, 1 ml of t-glutamine, and 1 ml of penicillin–streptomycin mixture. The cells were confluent (3.4 ± 0.83 × 10^5 cells/well) and cells between passages 4 and 8 were used. After 10 to 12 population doublings, the purity of the cultured astrocytes was assessed by immunostaining for glial fibrillary acidic protein.24 The number of viable cells was assessed after each experiment.

**Oxymyoglobin, Methemoglobin, and Hypoxia**

For preparation of oxymyoglobin, 6.5 ml of human donor blood was centrifuged at 2200 rpm for 10 minutes. After removal of plasma the packed red blood cells were washed three times with an equal volume of 0.9% NaCl. To lyze the red blood cells, five times the volume of double-distilled water was added. The solution was mixed for 3 minutes and centrifuged for 15 minutes at 2300 rpm. The supernatant of hemoglobin was removed and bubbled with O2 for 10 minutes. The solution was diluted 10 times with double-distilled water and the concentration of oxymyoglobin was determined by spectrophotometry. The hemoglobin concentration in solution was 230 μmol.

A solution of methemoglobin was prepared by oxidation of oxymyoglobin with a 1.2-fold molar excess of potassium ferricyanide followed by dialysis according to the method of Elliott and B. F.21 A cell culture incubator was modified to create a hypoxia chamber. An external tank of 11% oxygen, balanced with nitrogen, was connected to a line running inside the modified incubator. This gas line was connected to a small secondary chamber in which the cells were placed. The incubator was kept at 37°C for the duration of the experiment. The PO2 level at the medium was 50 to 89 mm Hg (control 143–169 mm Hg) under hypoxic conditions. Before the experiment, the normal cell medium was exchanged for a buffered medium consisting of DMEM with sodium bicarbonate replaced by 25 mM Na2-[hydroxyethyl]piperazine-N’-[ethanesulfonic acid] buffer, previously buffered to pH 7.4 with 1 N NaOH. The medium was degassed by vacuum and simultaneously bubbled with 100% N2 at 37°C for 30 minutes.
Endothelin-1 and vasospasm after SAH

**Results**

There were no significant changes in ET-1 levels in the perivascular space of the six monkeys from the microdialysis group that developed moderate vasospasm (36 ± 10% decrease of the AP area of the right MCA compared with preoperative values; mean ± standard deviation [SD]; range 25–50%) (Fig. 1 upper). Furthermore, the levels of ET-1 in plasma and CSF collected from the cisterna magna of the eight monkeys at the time of mild-to-moderate vasospasm of the right MCA (28 ± 7% decrease in the area of the right MCA compared with preoperative values; range 18–41%) did not change significantly compared with the ET-1 levels in plasma and CSF collected intraoperatively (Fig. 1 lower).

Transient global cerebral ischemia, during which regional CBF dropped by 37 ± 5% immediately after the ICA and CCA were clipped and maintained at this level for the 30 minutes of vessel occlusion, produced a significant increase in levels of ET-1 in CSF, but not in plasma. This effect started at 4 hours when the CSF ET-1 level increased to 3.1 ± 0.6 pg/ml and lasted up to 6 hours or more after reperfusion (Fig. 2). The ET-1 levels in CSF returned to normal 24 hours after reperfusion (0.7 ± 0.3 pg/ml in CSF and 0.4 ± 0 pg/ml in plasma) in the two monkeys that survived the experiment without neurological deficits.

**In Vitro Studies**

Control cultures of human brain endothelial cells and rat astrocytes had increasing ET-1 levels in the medium for the first 24 hours (Figs. 3 upper and center and 4). In the cultures of human brain endothelial cells, oxyhemoglobin produced a long-lasting (48-hour) decrease in ET-1 compared with the control cultures (p < 0.05) without affecting the number of viable cells (Fig. 3 upper).

In the control cultures of rat astrocytes, oxyhemoglobin inhibited ET-1 production (p < 0.05) (Fig. 3 center). This effect, however, was not specific to oxyhemoglobin, a vasospastic agent, because a solution of methemoglobin, a nonvasospastic agent, produced the same effect (Fig. 3 lower). The number of viable cells remained unchanged after exposure to the hemoglobins.

In the endothelial cell cultures, hypoxia initially pro-

---

**Sources of Supplies and Equipment**

In the in vivo studies, the semipermeable catheter used in the experiment on vasospasm and ET-1 in the perivascular space was purchased from Bioanalytical Systems, Inc., Lafayette, IN. The catheter was attached to a microosmotic pump, model 2ML1, obtained from Alza Inc., Palo Alto, CA. The contrast medium used in this experiment, Conray 60%, was provided by Mallinckrodt Medical Inc., St. Louis, MO, and the computerized image analysis system was Image 1.51, developed by Wayne S. Rasband, National Institute of Mental Health, Bethesda, MD. The CBF probe used in the experiment on cerebral ischemia and ET-1 in plasma and CSF was Saber thermomonitoring, obtained from Flowtronics, Phoenix, AZ.

In the in vitro studies, we used a Coulter counter, available from Coulter Electronics, Inc., Hialeah, FL, to count the number of viable cells. Dako Corp., Carpinteria, CA, provided the glial fibrillary acidic protein. Porcine and human ET-1 were obtained from Peninsula Laboratory, Inc., Belmont, CA.
duced a marked decrease of ET-1, which completely disappeared at 48 hours ($p < 0.05$) (Fig. 4 upper). In contrast, hypoxia in the astrocyte cultures produced an early burst of ET-1 production at 6 hours ($p < 0.05$) followed by a decrease in ET-1 production similar to that observed in the endothelial cells (Fig. 4 lower). In both cell lines, cell viability was retained at all time points.

**Discussion**

In addition to numerous oxyhemoglobin-related mechanisms,

In addition to numerous oxyhemoglobin-related mechanisms,

This controversial hypothesis is supported by a delayed onset of long-lasting spasm of the intracranial vessels produced by intracisternal administration of ET-1, by an increase in ET-1 levels in CSF after SAH in humans, and by a decrease in the incidence of vasospasm after use of ET-1 receptor antagonists.

Astrocytes, neurons, and pituitary cells produce ET-1. However, ET-1 is also released by endothelial and smooth-muscle cells when they are stimulated by oxyhemoglobin and by astrocytes when they are stimulated by thrombin. Thus, both the source and the mechanism of ET-1 presence in the subarachnoid space after SAH appear to be unclear. The goal of this study was to establish the cause and source of ET-1 presence in subarachnoid space in a primate model of SAH.

**Vasospasm and ET-1**

The results of our in vivo experiments indicate the con-
continuous presence of ET-1 in plasma, CSF, and the perivascular space in a primate model of SAH. The levels remained constant over the interval investigated, which included the typical period for vasospasm. The ET-1 levels in the perivascular space and in the cisternal CSF were high enough to produce vasospasm,

yet the levels were unchanged by the development or resolution of vasospasm. These results indicate that the increase in CSF ET-1 levels observed by others cannot be solely responsible for the development of vasospasm. On the other hand, if oxyhemoglobin, an agent purportedly responsible for vasospasm,

eliminates the action of vasodilatory agent(s), then vasospasm could result from the unopposed action of a constricting agent like ET-1. Recently, it was reported that nitric oxide (NO), a potent vasodilator,

is directly involved in the regulation of CBF. Moreover, a reversal of vasospasm by direct intracarotid infusion of NO solution indicates that there may be decreased availability of NO at the time of vasospasm. The combination of the continuous presence of low levels of ET-1 in the perivasculan space, such as those observed in our experiments, with decreased NO production due to the concomitant disappearance of NO synthase activity from the adventitia of the vessel or a decrease in NO availability due to a “sink effect” of hemoglobin, could be responsible for vasospasm. Because a decrease in NO availability results in increased ET-1 production, the decrease of vasospasm in response to ET-1 receptor antagonists as well as to NO may occur because both approaches tend to recover the normal balance between ET-1 and NO.

Our in vitro experiments did not confirm a relationship between oxyhemoglobin exposure and production of ET-1 by astrocytes or endothelial cells, as others have suggested. On the contrary, we found that production of ET-1 by the cells was inhibited by exposure to oxyhemoglobin or methemoglobin. The discrepancy between our results and those of others may be related to their use of a commercially available bovine double-crystallized hemoglobin (Sigma Chemical Co., St. Louis, MO), which is contaminated with hemin and endotoxins and which may be responsible for the increased production of ET-1 by the cells.

In summary, our experiments failed to identify an association between oxyhemoglobin and ET-1 production by astrocytic and endothelial cell cultures or between the level of ET-1 in CSF or the perivasculan space and vasospasm.

**Source and Cause of ET-1 Presence in CSF After Intracranial Aneurysm Rupture**

Although SAH from a ruptured intracranial aneurysm is usually treated as the extravasation of blood to CSF leading to vasospasm, it is a multifactorial event. Initially, SAH produces a dramatic increase in intracranial pressure that produces a temporary “stop-flow” phenomenon or at least partial ischemia during the first few minutes after rupture of the intracranial aneurysm. Elevation of ET-1 in the brain, plasma, and CSF occurs after cerebral ischemia. Transient cerebral ischemia in our model produced a prompt increase in the level of CSF ET-1, the timing of which was approximately the same as that induced by hypoxia in the in vitro experiments with astrocytes. Astrocytes, but not endothelial cells, responded to hypoxia with a brief burst of ET-1 production at 24 and 48 hours. The results indicate that the increase in CSF ET-1 levels observed by others cannot be solely responsible for the development of vasospasm. On the other hand, if oxyhemoglobin, an agent purportedly responsible for vasospasm, eliminates the action of vasodilatory agent(s), then vasospasm could result from the unopposed action of a constricting agent like ET-1. Recently, it was reported that nitric oxide (NO), a potent vasodilator, is directly involved in the regulation of CBF. Moreover, a reversal of vasospasm by direct intracarotid infusion of NO solution indicates that there may be decreased availability of NO at the time of vasospasm. The combination of the continuous presence of low levels of ET-1 in the perivascular space, such as those observed in our experiments, with decreased NO production due to the concomitant disappearance of NO synthase activity from the adventitia of the vessel or a decrease in NO availability due to a “sink effect” of hemoglobin, could be responsible for vasospasm. Because a decrease in NO availability results in increased ET-1 production, the decrease of vasospasm in response to ET-1 receptor antagonists as well as to NO may occur because both approaches tend to recover the normal balance between ET-1 and NO.

In summary, our experiments failed to identify an association between oxyhemoglobin and ET-1 production by astrocytic and endothelial cell cultures or between the level of ET-1 in CSF or the perivascular space and vasospasm.
not observe an increase in CSF ET-1 levels with this model, in contrast to the findings in patients. Our results suggest that the increased CSF ET-1 levels observed in patients after rupture of an intracranial aneurysm and in patients with clinical symptoms of vasospasm are not solely responsible for the development of vasospasm; rather, they result from ischemia occurring after SAH.

References

39. Norees H: The role of intracranial pressure in the arrest of hem-
Endothelin-1 and vasospasm after SAH


Address reprint requests to: Edward H. Oldfield, M.D., Surgical Neurology Branch, Building 10, Room 5D-37, National Institutes of Health, Bethesda, Maryland 20892.

Manuscript received November 21, 1996. Accepted in final form February 25, 1997.