Intracranial Heme Metabolism and Cerebral Vasospasm After Aneurysmal Subarachnoid Hemorrhage

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Background and Purpose—The goal of this prospective study was to clarify the potential role of an inducible heme-metabolizing enzyme, heme oxygenase (HO)-1, and an inducible iron-detoxifying protein, ferritin, in cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH).

Methods—The authors measured the levels of bilirubin and iron, which are by-products of HO-1, and the ferritin levels in the cerebrospinal fluid in 39 consecutive patients with aneurysmal SAH of Fisher computed tomography group III, and determined the relationship between these by-products of HO-1 or ferritin and vasospasm.

Results—Fourteen of 39 patients (35.9%) developed asymptomatic vasospasm, while 6 patients (15.4%) developed symptomatic vasospasm. The levels of ferritin, bilirubin, and iron were all significantly elevated after SAH. The levels of ferritin and bilirubin were significantly higher in patients with no vasospasm than in patients with asymptomatic and symptomatic vasospasm on days 5 through 7 (P<0.05, respectively) and on days 11 through 14 (P<0.025 in bilirubin) after SAH. However, no significant difference was observed in the iron levels between these patient groups.

Conclusions—This is the first study to show that higher levels of bilirubin and ferritin in the cerebrospinal fluid after SAH were associated with no vasospasm in clinical settings. These findings support the concept that the induction of HO-1 and ferritin may be an intrinsic regulatory mechanism that acts against cerebral vasospasm. (Stroke. 2003;34:2796-2800.)

Key Words: bilirubin ■ ferritin ■ iron ■ vasospasm, intracranial

Subarachnoid hemorrhage (SAH) causes excess quantities of iron in the perivascular space, which is a potentially toxic and pro-oxidant molecule, whether free or bound to heme. All available evidence suggests that the ferrous iron component of oxyhemoglobin (oxyHb) and deoxyhemoglobin (deoxyHb) may be responsible for cerebral vasospasm. Recent experimental studies reported that SAH induced heme oxygenase (HO)-1 and ferritin in cerebral arteries, which may be an intrinsic regulatory mechanism that acts against cerebral vasospasm. HO-1 is an inducible isozyme of HO, the rate-limiting enzyme in the heme degradative pathway, which metabolizes heme in hemoglobin (Hb) and generates carbon monoxide, free iron (ferrous iron), and biliverdin (subsequently reduced to bilirubin). The released iron stimulates ferritin synthesis, which ultimately provides an iron detoxification mechanism. This ferritin-mediated iron detoxification following heme metabolism may account for the antivasospastic effect observed after the induction of HO-1 and ferritin, although other mechanisms are also possible. However, if inadequate induction of HO-1 and ferritin contributes to the pathogenesis of vasospasm in clinical addition to experimental settings, patients with vasospasm will have lower bilirubin and ferritin in the cerebrospinal fluid (CSF). To test this hypothesis, we measured iron, bilirubin, and ferritin levels in the CSF obtained from SAH patients and determined the relationship between these by-products of HO-1 or ferritin and vasospasm in this study.

Materials and Methods

The present study was approved by the ethical committee of our institute and was performed in accordance with institutional guidelines. Appropriate informed consent was obtained from patients or their relatives.

Patient Population

This prospective study included 39 consecutive patients (17 male and 22 female), 38 to 90 years of age (mean 60.4 years), all with aneurysmal SAH of Fisher computed tomography (CT) group III, who were admitted to our institute between June 1, 1999, and June 30, 2002. Excluded from the study were patients who suffered from any angiographic or surgical complication and patients who did not undergo stable xenon CT (Xe-CT) examination or had a motion artifact on the Xe-CT scan. Patients with acute hydrocephalus who were treated with ventricular drainage were also excluded, because any drainage may change the CSF dynamics that have an influence on the measured parameters. World Federation of Neurosurgical Societies (WFNS) SAH scores at admission evaluated 14 patients as grade I, 10 as grade II, 3 as grade III, 7 as grade IV, and 5 as grade V. The ruptured aneurysm location was the anterior communicat-
ing artery in 13 patients, middle cerebral artery in 11, posterior communicating artery in 12, anterior cerebral artery in 2, and posterior inferior cerebellar artery in 1.

After angiographic confirmation of the aneurysm, surgical clipping (38 patients) or endovascular coiling (1 patient) of the lesion was performed within 48 hours of the initial onset. Neither cisternal nor lumbar drainage was utilized. All patients received intravenous fasudil hydrochloride from 1 day postsurgery to day 14 posthemorrhage. Additional treatment was administered to maintain normovolemia, prevent pneumonia and hypoxia, and correct anemia and hypoproteinemia.

Transcranial Doppler (TCD) sonography was performed daily. A Xe-CT examination was performed at least once on days 7 through 9 after onset, in all patients even if vasospasm was not suggested, and was also performed when the clinical examination or blood velocity as measured by TCD sonography suggested vasospasm. The resting cerebral blood flow (CBF) measurements were made with a Xe-CT CBF system (AZ-725, AZ-7000W, Anzai Medical Co, Ltd) adapted to a CT scanner (X-vision, Toshiba Corp) according to the manufacturer’s protocol. Two axial CT scan slices were examined for the CBF study: one included the basal ganglia and the other the body of the lateral ventricles. CBF data were analyzed and the mean CBF in all vascular territories was determined as described by Yonas et al.13 CT scanning was performed to evaluate all instances of clinical deterioration. All patients with symptomatic vasospasm were treated with hypertensive hypervolemic therapy.

Symptomatic vasospasm was defined as otherwise unexplained (1) clinical deterioration (ie, a new focal deficit, decrease in the level of consciousness, or both) or (2) a new infarct on CT that was not visible on the admission or immediate postoperative scan, or both. Other potential causes of clinical deterioration, such as hydrocephalus, rebleeding, or seizures, were rigorously excluded. Asymptomatic vasospasm was defined as regional hypoperfusion (<80% of CBF in other regions) identical with vascular territories on the Xe-CT scan but no association with clinical deterioration or a new infarct as described above. Evidence of vasospasm by angiography was generally used to support the diagnosis but was not mandatory. The clinical outcome was evaluated using the Glasgow Outcome Scale at 3 months after onset.14

CSF Analysis
CSF samples were obtained via a lumbar tap on days 5 through 7, 8 through 10, and 11 through 14 after onset. The CSF sampling was performed only once during the interval in each patient and the day for sampling was randomly selected. Control CSF samples were obtained from 10 patients with minimal cervical or lumbar spondylosis without myelopathy or radiculopathy at computed tomographic myelography. The concentrations of total proteins and inflammatory cells in the CSF were determined with the aid of an automatic chemistry analyzer. CSF samples were analyzed at an outside laboratory (BML, Inc) for the levels of iron, bilirubin, and ferritin, which were determined using a quick-auto-neo-Fe (K) (Shino-Test Corp), a total bilirubin E-HR-Wako (Wako Pure Chemicals Industries, Ltd), and an Immunoticles Auto-ferritin 2 (A & T Corp), respectively.

Statistical Analysis
All values were expressed as mean±standard error of the mean. Comparisons between the 2 groups were made by unpaired t tests, and intergroup comparisons among the 3 or more groups were determined by 1-way analysis of variance and then the Tukey-Kramer multiple comparison procedure (95% lower and upper confidence interval) if significant variance was found. A P value of <0.05 was considered statistically significant.

Results
Incidence of Vasospasm
Twenty of 39 patients (51.3%) developed regional hypoperfusion identical with vascular territories on the Xe-CT scan, 14 of whom (35.9%) developed asymptomatic vasospasm, and 6 of whom (15.4%) developed symptomatic vasospasm. Patients with asymptomatic and symptomatic vasospasm were combined into a vasospasm group. Thus, the vasospasm group contained 20 patients and the nonvasospasm group contained the remaining 19 patients.

Ferritin Concentrations in the CSF
The CSF levels of ferritin in patients with SAH (1526.3±251.3 ng/mL) were a few hundred-fold higher than in control patients (6.0±0.9; P<0.025). The ferritin levels slightly increased from days 5 through 7 to days 8 through 10, and thereafter decreased (Figure 1). The ferritin levels in the nonvasospasm group were higher than in the vasospasm group, whose difference reached significance at days 5 through 7 (P<0.05). The ferritin levels in asymptomatic and symptomatic vasospasm cases were similar.

Bilirubin Concentrations in the CSF
The CSF levels of total bilirubin in patients with SAH (0.5±0.06 mg/dL) were approximately 10-fold higher than in control patients (0.06±0.005; P<0.025). The bilirubin levels gradually decreased from days 5 through 7 to days 11 through 14 (Figure 2). The bilirubin levels in the nonvasospasm group...
were higher than in the vasospasm group, whose difference reached significance at days 5 through 7 (P<0.05) and days 11 through 14 (P<0.025). The bilirubin levels in asymptomatic and symptomatic vasospasm cases were also similar.

**Iron Concentrations in the CSF**
The CSF levels of iron in patients with SAH (27.9±3.9 μg/dL) were approximately 10-fold higher than in control patients (2.3±0.7; P<0.025). The iron levels were almost unchanged from days 5 through 7 to days 11 through 14. No significant difference was observed between the vasospasm and the nonvasospasm groups.

**Inflammatory Cell and Total Protein Concentrations in the CSF**
The concentration of inflammatory cells and total protein in the CSF markedly increased after SAH and gradually decreased from days 5 through 7 to days 11 through 14. There was no significant difference in the concentration of inflammatory cells and total protein between the vasospasm and the nonvasospasm groups.

**Neurological Grades at Admission and Clinical Outcome**
With regard to the WFNS grades at admission, the bilirubin levels in patients with grade V were significantly higher than in patients with grades II (P<0.005) and IV (P<0.025). There were no significant differences in the levels of ferritin and iron among the WFNS grades at admission. The levels of ferritin and bilirubin were higher in the nonvasospasm group than in the vasospasm group irrespective of the WFNS grades at admission, but the iron level was not.

The clinical outcome showed that 27 patients achieved good recovery (including 11 with asymptomatic vasospasm and 2 with symptomatic vasospasm), 6 had moderate disability (including 2 with asymptomatic vasospasm and 1 with symptomatic vasospasm), 5 had severe disability (including 1 with asymptomatic vasospasm and 3 with symptomatic vasospasm), and 1 patient had a persistent vegetative state. The levels of ferritin and bilirubin in patients with good recovery were significantly lower than in patients with moderate disability (P<0.01 and P<0.005, respectively); however, the ferritin and bilirubin levels were higher in the nonvasospasm group than in the vasospasm group at any clinical outcome. There were no significant differences in the iron levels among the clinical outcomes.

**Discussion**
The novel findings of the present study were that the patients with no vasospasm were associated with higher levels of ferritin and bilirubin, one of the HO-1 metabolites, in the CSF after SAH of Fisher CT group III. It is notable that a significant change in both levels was observed during the early phase of vasospasm at days 5 through 7. The iron levels were not significantly different between the vasospasm and the nonvasospasm groups, although the present method measuring iron did not allow the differentiation of ferritin-bound iron from other non–heme-bound iron. These findings suggest that a faster and greater rate of Hb breakdown and ferritin-mediated iron detoxification might decrease the redox-active iron and therefore prevent the occurrence of vasospasm. These findings support the concept that the induction of HO-1 and ferritin may be an intrinsic regulatory mechanism that acts against cerebral vasospasm.

A major factor that mediates cerebral vasospasm was reported to be oxyHb and deoxyHb, which spontaneously oxidize to methemoglobin (metHb) and release free radicals.1-3 In the subarachnoid space, erythrocytes, oxyHb, deoxyHb, and metHb are removed via the arachnoid villi9 or phagocytosis by cells of the pial membrane and free subarachnoid macrophages,17 which induce HO-1 and metabolize heme.6 However, heme, which is easily released from metHb and accumulates in SAH, is very hydrophobic and associates preferentially with membrane components, preventing its rapid removal from the CSF.1-18 If heme accumulates in the erythrocyte membranes, it causes hemolysis and promotes a release of oxyHb and deoxyHb into the subarachnoid space.1 The best defense against heme toxicity appears to be its rapid degradation by HO-1; inflammatory, pial membrane, brain glial, and vascular cells may all play a role in metabolizing heme.7,19 Thus, HO-1 may perform important in vivo antioxidant and antivasospasm functions purely by depletion of deleterious heme in SAH.7,20

On the other hand, the ability of cells expressing HO-1 to decrease intracellular iron content may be particularly important after SAH, because cells are exposed to high levels of heme that increase HO-1 activity, thus generating high levels of intracellular free iron. This iron enhances the synthesis of the iron storage protein ferritin, which may participate in a protective role against toxic effects of iron overload in cells.21 Hemin itself, oxidative stress, or inflammatory cytokines may also be involved in an iron-independent induction of ferritin.22 The potent antioxidant efficiency provided by ferritin probably depends on one or both of its high sequestering capacity for inorganic iron and the intrinsic ferroxidase activity of its H chain that converts ferrous iron to ferric iron.21 Relatively soluble ferrous iron is incorporated into the ferritin shell much more efficiently than ferric iron,22 and ferritin-stored iron may resist cyclical reduction/oxidation reactions that tend to propagate and amplify oxidative damage. Furthermore, rapid induction of ferritin may paradoxically reduce the levels of free iron as more becomes sequestered.23 The apparent cytoprotective effects of HO-1 may occur only when both the free heme has been destroyed and the free iron has been completely sequestered into the ferritin.

Detoxification and transfer of the redox-active iron may be important in CSF as well within the cells, because most iron may leave CSF by the bulk drainage of CSF via the arachnoid villi and other routes,24 being recycled to the blood plasma.25 HO-1 plays a key role in recycling Hb iron, and a link between iron release from the cells and HO-1 activity is suggested.22,26 Free iron is also released from methHb and heme by hydrogen peroxide.14 Mori et al14 reported evidence for enhanced production of ferrous or ferric iron ions in the subarachnoid space after SAH. On the other hand, CSF in itself has a low iron-binding capacity that is close to saturation under normal conditions.27 Although citrate, ascorbate, ceruloplasmin, and albumin may influence the state of iron in...
CSF, non–transferrin-bound iron in CSF is present in the ferrous state.\textsuperscript{24} Ferritin, another protein with a marked iron-binding capacity, is primarily localized intracellularly\textsuperscript{21} and its concentration in CSF is very low,\textsuperscript{25,28} although the relatively high amount of iron can be delivered via ferritin in CSF.\textsuperscript{29} However, in pathological conditions such as SAH, induced ferritin not only protects intracellular targets but is also secreted into the CSF, where it might exert protective effects in extracellular locations.\textsuperscript{30} In the present study, ferritin increased more than 10-fold greater than iron in the CSF after SAH, and higher CSF levels of ferritin were observed in patients with no vasospasm. These findings suggest that ferritin may be the primary iron delivery protein and may act against cerebral vasospasm by detoxifying the free iron in the CSF after SAH.

The effects of bilirubin on cerebral vasospasm are unknown. Findings that oxyHb or deoxyHb causes vasospasm whereas metHb does not disagree with the theory that bilirubin is an important spasmogen, since each type of Hb produces bilirubin in the subarachnoid space.\textsuperscript{3} Bilirubin has the potential to act as a free radical scavenger and chain-breaking antioxidant, which may act against vasospasm.\textsuperscript{3,31} Recent experimental studies showed that the induction of HO-1, a bilirubin-generating enzyme, prevented vasospasm.\textsuperscript{7,15} In contrast, another recent experimental study showed that bilirubin-oxidation products might contribute to or cause cerebral vasospasm after SAH.\textsuperscript{32} In the present study, higher CSF levels of bilirubin in addition to ferritin were observed in patients with no vasospasm. Possible explanations for these finding are as follows: (1) bilirubin itself may not be an important spasmogen; and (2) the increased heme metabolism and the ferritin-mediated iron detoxication may decrease the redox-active iron, resulting in the higher bilirubin levels but in the lower bilirubin-oxidation product levels. The conditions in which bilirubin is oxidized, such as an iron-dependent free radical chain mechanism, may be indispensable for the occurrence of vasospasm.\textsuperscript{1–4}

This study had some limitations. First, the definition of asymptomatic vasospasm depended on the Xe-CT without mandatory angiography, although angiography was performed for equivocal cases. It might be possible to miss patients with bilateral or diffuse vasospasm, or vasospasm of a degree that did not produce an 80% decrease in CBF, while containing patients with poor clinical grades, in whom the detection of a clinical worsening was difficult. The CBF measurement also had some limitations, as other factors such as surgical manipulations could cause a decrease in the regional CBF. Second, the volume of SAH might be variable, although the SAH of this study was limited to the Fisher CT group III. The SAH in patients with the WFNS grade V at admission might be more severe, resulting in their bilirubin levels becoming significantly higher. In contrast, the patients with less-severe SAH, that might produce lower levels of ferritin and bilirubin, might achieve good recovery. Another possible explanation for lower ferritin and bilirubin levels in patients with good recovery is that their SAH might be removed by the bulk drainage of CSF via the arachnoid villi rather than being intracranially metabolized. Third, the present findings only support and did not confirm that up-regulation of HO-1 and ferritin mitigates against vasospasm. Because HO-1 acts intracellularly and is not known to be secreted extracellularly,\textsuperscript{9} we did not measure HO-1 levels but only measured its metabolites in the CSF in a relatively small number of patients.

In conclusion, this is the first study to show that higher levels of bilirubin, a metabolite of HO-1, and ferritin in the CSF after SAH were associated with no vasospasm in clinical settings. HO-1 and ferritin are important for mammalian iron homeostasis and for rapid protection of cells from potential oxidative damage.\textsuperscript{20,26} Thus, removal or detoxification of both free and heme-bound iron may be critical in antioxidant and antivasospasm strategy. Therapeutic induction of HO-1 and ferritin may prove to be a novel approach for the prevention and treatment of cerebral vasospasm.\textsuperscript{15}

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References

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