Plasma endothelin concentrations after aneurysmal subarachnoid hemorrhage

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Object. The pathogenesis of cerebral vasospasm and delayed ischemia after subarachnoid hemorrhage (SAH) seems to be complex. An important mediator of chronic vasospasm may be endothelin (ET), with its powerful and long-lasting vasoconstricting activity. In this study the author investigated the correlation between serial plasma concentrations of ET and ischemic symptoms, angiographically demonstrated evidence of vasospasm, and computerized tomography (CT) findings after aneurysmal SAH.

Methods. Endothelin-1 immunoreactivity in plasma was studied in 70 patients with aneurysmal SAH and in 25 healthy volunteers by using a double-antibody sandwich-enzyme immunoassay (immunometric) technique.

On the whole, mean plasma ET concentrations in patients with SAH (mean ± standard error of mean, 2.1 ± 0.1 pg/ml) did not differ from those of healthy volunteers (1.9 ± 0.2 pg/ml). Endothelin concentrations were significantly higher (p < 0.05) in patients who experienced delayed cerebral ischemia with fixed neurological deficits compared with those in other patients (post-SAH Days 0–5, 3.1 ± 0.8 pg/ml compared with 2.1 ± 0.2 pg/ml; post-SAH Days 6–14, 2.5 ± 0.4 pg/ml compared with 1.9 ± 0.2 pg/ml). Patients with angiographic evidence of severe vasospasm also had significantly (p < 0.05) elevated ET concentrations (post-SAH Days 0–5, 3.2 ± 0.8 pg/ml; post-SAH Days 6–14, 2.7 ± 0.5 pg/ml) as did those with a cerebral infarction larger than a lacuna on the follow-up CT scan (post-SAH Days 0–5, 3.1 ± 0.8 pg/ml; post-SAH Days 6–14, 2.5 ± 0.4 pg/ml) compared with other patients. Patients in whom angiography revealed diffuse moderate-to-severe vasospasm had significantly (p < 0.05) higher ET levels than other patients within 24 hours before or after angiography (2.6 ± 0.3 compared with 1.9 ± 0.2 pg/ml). In addition, patients with a history of hypertension or cigarette smoking experienced cerebral infarctions significantly more often than other patients, although angiography did not demonstrate severe or diffuse vasospasm more often in these patients than in others.

Conclusions. Endothelin concentrations seem to correlate with delayed cerebral ischemia and vasospasm after SAH. The highest levels of ET are predictive of the symptoms of cerebral ischemia and vasospasm, and ET may also worsen ischemia in patients with a history of hypertension. Thus, ET may be an important causal or contributing factor to vasospasm, but its significance in the pathogenesis of vasospasm remains unknown.

Key Words • delayed cerebral ischemia • endothelin • subarachnoid hemorrhage • vasospasm • aneurysm

Aneurysmal SAH is a serious disease that carries high mortality (40–50%) and morbidity rates despite recent improvements in surgical and medical treatments.33–35 Delayed cerebral ischemia, commonly attributed to vasospasm in large cerebral arteries, causes death or disability that occurs after the primary hemorrhage.22–59

The pathogenesis of cerebral vasospasm and delayed ischemia after SAH is unclear and seems to be multifactorial;35,52,59 therefore, current treatment modalities may appear unsatisfactory. However, use of calcium channel blockers (nimodipine, nicardipine, and AT877), hypertension and/or hypervolemia therapy, and endovascular vasospasm treatments (transluminal angioplasty and superselective intraarterial papaverine infusion) have been shown to decrease the incidence or severity of delayed ischemia, mainly by improving the rheological properties of cerebral circulation.55,59,57

Endothelin-1, a 21-amino acid peptide, has a potent and long-lasting vasoconstricting activity that is mediated by an increase in the intracellular concentration of calcium.32–34,63,65 Intracisternal injections of ET-1 mimic the angiographic pattern and morphological changes observed during cerebral vasospasm better than other vasoconstrictors.26,65 Endothelin-1 is thus a potential candidate for an important causal factor in delayed arteriospasm after SAH. However, conflicting reports exist concerning ET concentrations in CSF and in plasma after SAH, as well as the effect of ET receptor antagonists on post-SAH cerebral vasospasm.55,53,52,65 This lack of agreement may result from the different methods used in each study, which is likely due to a lack of statistical power from these small sample sizes. In addition, an increase in ET-1 levels in
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CSF after SAH may result from a release of ET-1 from astrocytes following cerebral ischemia and, thus, may be the result of cerebral ischemia rather than the cause of cerebral vasospasm. Elevated plasma ET-1 concentrations have been found to correlate with the severity of different vascular diseases, and these concentrations also may reflect endothelial dysfunction or damage.

In this study, the association of plasma ET immunoreactivity with various factors—elapsed time after SAH, surgery, ischemic symptoms, radiological findings, and outcome—was investigated in a relatively large patient population. The goal was to find significant relationships and to rule out the effect of confounding factors.

Clinical Material and Methods

Patient Population

This prospective study focused on 70 patients (36 men and 34 women; age 24–65 years [median age 45.8 years]) with aneurysmal SAH who were admitted within 96 hours after onset of bleeding to the Department of Neurosurgery, Helsinki University Central Hospital. A control population of 25 healthy volunteers (11 men and 14 women; age 22–55 years, [median age 40.1 years]) was also examined. The healthy volunteers denied having received any medication for 1 week before plasma sampling.

Patients were considered to have hypertension if they used antihypertension medications or if their pre-SAH blood pressure readings had repeatedly exceeded 160/96 mm Hg (systole/diastole). There were 23 patients (33%) with a history of hypertension, 10 (14%) of whom used antihypertension medications. Two SAH patients (3%) had diabetes, 44 (63%) currently smoked cigarettes, and 13 (19%) heavily drank alcohol.

Clinical Monitoring, Treatment, and Patient Outcome

Each patient’s clinical condition on admission and before surgery was graded according to the Hunt and Hess Scale. The presence of SAH was verified using CT scanning or by lumbar puncture and operation. The amount of subarachnoid blood seen on CT scans—all of which were acquired within 48 hours after SAH—was categorized according to the scale developed by Fisher, et al. (Table 1). Computerized tomography scans were again obtained at discharge from the hospital, during the follow-up examination, and earlier if clinical deterioration occurred. In every patient the aneurysmal origin of the SAH was confirmed.

Of the 70 patients, 52 (74%) underwent surgical clipping of the ruptured aneurysm. Four patients with a decreased level of consciousness underwent surgery on an emergency basis because they had a space-occupying intracerebral hematoma. The majority of the surgically treated patients (47 [90%]) were in good clinical condition (Hunt and Hess Grades I–III) and underwent surgery to prevent rebleeding.

Nimodipine treatment was started after diagnosis of a ruptured aneurysm and was continued for up to 21 days after SAH. Surgically treated patients received betamethasone routinely, 4 mg every 6 hours, starting the day before surgery and continuing to the 6th postoperative day with diminishing doses. No hypertension, hypervolemia, or endovascular vasospasm therapy was used.

Neurological examinations were performed daily after admission. Delayed cerebral ischemia was determined as a gradual development of focal neurological signs and/or deterioration in the level of consciousness not due to intracerebral hematoma, rebleeding, hydrocephalus, clipping of an arterial branch together with the aneurysm, in-
fection, serum electrolyte disorders, or any other known reason. Causes of poor clinical condition and outcome were determined using repeated CT scanning, routine postoperative angiography, autopsy, or laboratory investigations.

Both severity and distribution of vasospasm were determined by examining postoperative angiograms obtained 5 to 14 days after onset of SAH. Vasospasm was graded as severe if the vessel lumen was narrowed to 50% or less of its original diameter (measurements obtained during preoperative angiography performed shortly after admission), moderate for 30 to 49% narrowing, and none or mild for less than a 30% compromise of the lumen. Moderate or severe vasospasm was considered local if it involved only one or a portion of one major vascular distribution, and diffuse if it involved more than one vascular distribution.

Patient outcome was assessed at 1 year post-SAH according to the Glasgow Outcome Scale.21 Follow-up CT scans were obtained at this time from 49 patients to identify permanent hypodense lesions consistent with cerebral infarction. Such lesions had not been seen on CT scans obtained immediately after patient admission, and their location ruled out previous intracerebral hematoma or a lesion caused by clipping the arterial branch that contained the aneurysm. In 14 patients who died within 1 year after SAH or in whom a CT scan could not be obtained at the 1-year follow-up examination, the follow-up CT scan was considered to be any image that had been obtained more than 3 weeks after SAH to find permanent hypodense lesions.

Hypodense lesions could be visualized on CT scans in 28 patients; they were not found in 35. Causes of the hypodense areas were grouped as follows: 1) a lesion in the same areas as a previous intracerebral hematoma (seven patients) or a lesion caused by clipping the arterial branch that contained the aneurysm (one patient); and 2) a lesion caused by delayed cerebral ischemia (20 patients). If patients had separate hypodense areas caused by both delayed ischemia and previous intracerebral hematoma, they were included in the delayed ischemia group. The hypodense areas caused by delayed cerebral ischemia that was consistent with cerebral infarctions were further categorized according to their size, shape, and localization.

All small (< 20 mm in diameter) hypodense areas were consistent with lacunar infarctions were located in the basal ganglia (five patients), thalamus (two patients), or subcortex (two patients), and were ovoid or round. The larger (> 20 mm in diameter) hemispheric hypodense areas exhibited a typical wedge shape corresponding to their vascular distribution and were situated in the subcortical white matter and/or the cerebral cortex (11 patients).

**Laboratory Procedures**

A total of 320 plasma samples were collected from the 70 SAH patients on weekdays from the 1st day after admission until hospital discharge or death, at a frequency of one sample every 1 to 4 days. The number of samples per patient varied from one to eight. Only one sample was collected from each healthy volunteer.

For studying plasma ET-1 immunoreactivity, a double-antibody sandwich-enzyme immunoassay (immunometric), described in detail elsewhere,28 was performed using a commercially available kit (Cayman Chemical Co., Ann Arbor, MI). Briefly, after patients or volunteers had fasted overnight, blood samples (10 ml) were collected with minimal stasis from an antecubital vein into tubes containing potassium–ethylenediamine tetraacetic acid (final concentration 2 mg/ml) and aprotinin (TrasyloL; Miles Laboratories, Elk hart, IN; final concentration 500 IU/ml). Plasma was prepared by centrifugation of blood at 4°C for 15 minutes at 1000 G, and samples were stored at −70°C until assayed.

Endothelin was extracted from plasma by using Seppak C18 cartridges (Waters Associates, Milford, MA). Results were corrected for an extraction recovery rate of 85%. Endothelin standards and samples were incubated at 4°C in the wells of a microtiter plate, coated with a monoclonal ET-specific (immobilized) antibody, and kept overnight with ET antibody–acyethylcholinesterase conjugate, an enzyme-labeled antibody tracer. After incubation, excess reagents were washed away and the wells were rinsed with a buffer solution. Thereafter, Ellman’s reagent (acetlythiocholine + 5.5’-dithio-bis-[2-nitrobenzoic acid]) was added to each well. Hydrolysis of acetylthiocholine by acetylcholinesterase produces thiocholine. A nonenzymatic reaction of thiocholine to 5,5’-dithio-bis-[2-nitrobenzoic acid] produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. Plates were examined after a development time of 120 minutes and the results were measured spectrophotometrically and calculated from the standard curve. According to the manufacturer, the intra- and interassay coefficients of variation are less than 10%. Crossreactivity was 100% for ET-2, 66% for ET-3, and less than 0.01% for big ET. Samples were analyzed by a laboratory technician who had no knowledge of the patients’ case histories.

**Statistical Analysis**

Because samples from different patients were collected on different post-SAH days, the data were reorganized using a data-manager program and analyzed using a biomedical data package statistical program (version 1993, release 7.0; BMDP Statistical Software, Inc., University of California, Los Angeles, CA), so that for each patient the results for samples obtained on the day closest to the 2nd (range 1–3 days; 44 patients), 5th (4–6 days; 50 patients), 8th (7–9 days; 43 patients), 12th (10–14 days; 45 patients), 18th (15–20 days; 32 patients), and 25th (22–28 days; 16 patients) days after SAH were included in the preliminary statistical comparisons. Because ET values remained quite similar throughout the hospital stay and to get more power for statistical comparisons, the data were reorganized for final statistical comparisons so that the results for samples taken on the day closest to the 3rd (range 1–5 days; 63 patients), 9th (6–14 days; 61 patients), and 19th (15–28 days; 34 patients) days after SAH were included in the statistical comparisons.

Categorical variables were compared using Fisher’s exact two-tailed test or Pearson’s chi-square test. Continuous variables were compared between groups by using the Mann–Whitney U-test or Student’s t-test or by ANOVA with corrected multiple pairwise comparisons based on

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either Bonferroni’s or Dunnett’s method. In patients for whom there were no missing ET data, the effect of elapsed time after SAH and different grouping variables on ET levels (expressed as the mean ± SEM) were compared using repeated-measures ANOVA and analysis of covariance. For analyses of both variance and covariance, ET values were analyzed after logarithmic transformation to obtain equality of variances between different groups. A two-tailed probability value less than 0.05 was considered to indicate statistical significance.

Results

Clinical Variables, Radiological Findings, and Outcome

In Table 1 is listed detailed information on clinical variables, radiological findings, and outcomes in the 70 patients with aneurysmal SAH. In 66 patients, symptoms of delayed cerebral ischemia could be estimated. Delayed ischemia with FND was highly correlated (p < 0.01) with the amount of subarachnoid blood as well as with the presence of a hypodense area on the follow-up CT scan (Table 2). Delayed cerebral ischemia with FND proved to be an important cause of deterioration and morbidity: 14 of 17 patients who were classified as having a moderate or severe disability or as being in a vegetative state at 1 year post-SAH experienced delayed ischemia with FND.

Of 13 patients in whom angiography demonstrated severe vasospasm, follow-up CT scan demonstrated a hypodense lesion attributable to vasospasm in nine (69%). Five (42%) of 12 patients in this group of 13 had delayed ischemia with FND, whereas only seven (19%) of 36 patients with less severe vasospasm had such a lesion. In this group of 36 patients, five (15%) of 34 had cerebral ischemia with FND.

Follow-up CT scans revealed a hypodense lesion due to delayed ischemia more often in patients with diffuse moderate-to-severe vasospasm than in patients with no vasospasm or localized vasospasm (50% compared with 25%, respectively); this was also the case with delayed ischemia accompanied by FND (38% in patients with diffuse moderate-to-severe vasospasm compared with 13% in patients with no vasospasm or localized vasospasm).

Computerized tomography scanning also revealed hypodense lesions due to delayed ischemia more often in those patients who smoked cigarettes before onset of SAH than in those who did not (40% compared with 14%; p < 0.05) and in patients who were hypertensive prior to hemorrhage compared with those who were not (55% compared with 21%, respectively; p < 0.01). Patients with a history of hypertension and cigarette smoking more often had delayed cerebral ischemia with FND (p = 0.012 and p = 0.08, respectively). Although angiography revealed that patients with hypertension had severe vasospasm more often than patients without hypertension (46% compared with 19%; not significant), patients with a history of hypertension experienced diffuse vasospasm less often (31% compared with 36%, respectively). A history of smoking before SAH had no effect on the occurrence of either severe (29% compared with 21%) or diffuse (34% compared with 36%) vasospasm when compared with angiographic findings for patients who did not smoke.

TABLE 2
Association of delayed cerebral ischemia with various factors in 66 patients with aneurysmal SAH*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Absent (%)</th>
<th>Present (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>amount of subarachnoid blood (66 patients)†</td>
<td>no blood or thin layer</td>
<td>23 (96)</td>
<td>1 (4)</td>
</tr>
<tr>
<td></td>
<td>thick layer</td>
<td>28 (67)</td>
<td>14 (33)</td>
</tr>
<tr>
<td>hypodense area (59 patients)‡</td>
<td>no hypodense area</td>
<td>32 (94)</td>
<td>2 (6)</td>
</tr>
<tr>
<td></td>
<td>Group 1</td>
<td>6 (100)</td>
<td>0 (0)</td>
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<td></td>
<td>Group 2</td>
<td>3 (33)</td>
<td>6 (67)</td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>3 (30)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>history of hypertension (66 patients)§</td>
<td>no</td>
<td>39 (87)</td>
<td>6 (13)</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>12 (57)</td>
<td>9 (43)</td>
</tr>
</tbody>
</table>

* In four patients occurrence of delayed ischemia could not be determined; in an additional seven patients no CT findings on hypodense area were available.
† p = 0.0065 for association between amount of subarachnoid blood and delayed cerebral ischemia.
‡ p < 0.001 for association between hypodense area groups and delayed ischemia.
§ p = 0.012 for association between history of hypertension and delayed ischemia.

Plasma ET Concentrations in Patients and Healthy Volunteers

On the whole, plasma ET immunoreactivity did not differ between samples obtained from SAH patients (2.1 ± 0.1 pg/ml; 320 samples) and those from healthy volunteers (1.9 ± 0.2 pg/ml; 25 samples). Both pre- and postoperative samples were available for 43 SAH patients. Surgery did not seem to affect plasma ET levels because preoperative samples (collected on the day of or 1 day before surgery) and postoperative samples (1–4 days [mean ± SD, 2.7 ± 1.7 days] after the operation) showed similar values for plasma ET-1 immunoreactivity (2.2 ± 0.2 pg/ml).

Comparison of Plasma ET Levels and Time After SAH

Among SAH patients, there seemed to be no association between plasma ET values and time elapsed since hemorrhage. Across time, plasma ET values were as follows: 2.3 ± 0.3 pg/ml within 1 to 3 days after SAH (44 patients); 2.1 ± 0.2 pg/ml within 4 to 6 days (50 patients); 2.1 ± 0.2 pg/ml within 7 to 9 days (43 patients); 2.2 ± 0.2 pg/ml within 10 to 14 days (45 patients); 2.2 ± 0.2 pg/ml within 15 to 21 days (32 patients); and 2.0 ± 0.3 pg/ml within 22 to 28 days (16 patients) after SAH.

Comparison of Plasma ET Levels and Delayed Ischemia and Time After SAH

An examination of plasma ET levels as they relate to occurrence of delayed cerebral ischemia with FND, hypertension, and time elapsed after SAH is shown in Table 3. Patients who had delayed ischemia with FND had significantly elevated ET values during the first 2 weeks after SAH, and these levels decreased significantly (p < 0.05) over time. There was no significant interaction between

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delayed ischemia and time after SAH on the ET levels. The ET levels were not significantly elevated by a history of hypertension (Table 3). Plasma levels were also higher (p = 0.065; nearly significant) in patients with delayed ischemia after adjustment for a history of hypertension.

In addition, outcome associated with ET levels after patients experienced delayed cerebral ischemia was the main reason for a dependent state (severe disability or vegetative state) at 1 year, although delayed ischemia was not the most important cause of death. Patients in a dependent state had significantly (p = 0.036) higher ET levels than those who were independent at 1 year post-SAH (Days 0–5; 3.6 ± 1 compared with 2 ± 0.2 pg/ml; Days 6–14, 2.8 ± 0.5 compared with 1.9 ± 0.2 pg/ml). Among those patients who died, the corresponding values were 1.8 ± 0.2 and 1.8 ± 0.3 pg/ml, respectively.

Plasma ET Levels, Hypodense Lesions, and Angiographic Evidence of Vasospasm

A comparison of ET levels according to the presence of a hypodense lesion on the follow-up CT scan and angiographic evidence of vasospasm are shown in Table 4. Hypodense lesions due to delayed cerebral ischemia as well as severe vasospasm angiographically revealed were significantly (p < 0.05)—and diffuse vasospasm was nearly significantly—associated with increased ET levels during the first 2 weeks after SAH. Adjustment for a history of hypertension reduced these significant associations, except for the association between ET levels and diffuse vasospasm demonstrated on angiography, which became significant.

In addition, ET values in samples obtained within 24 hours either before or after angiography were higher in patients with angiographic evidence of severe vasospasm (2.7 ± 0.4 pg/ml in 15 patients compared with 2 ± 0.2 pg/ml in 34 patients; p = 0.078); the values were also higher in patients with angiographic evidence of diffuse moderate-to-severe vasospasm (2.6 ± 0.3 pg/ml in 17 patients compared with 1.9 ± 0.2 pg/ml in 30 patients; p = 0.048) than in other patients. Patients with diffuse vasospasm also had significantly (p = 0.039) higher plasma ET-1 levels when a history of hypertension was taken into account. No significant associations were found between plasma ET-1 levels and patient’s sex, age, amount of subarachnoid bleeding, reversible ischemic neurological deficit, clinical condition on admission, and cigarette smoking (data not shown).

Discussion

Endothelium Injury and Vasospasm

Chronic post-SAH vasospasm seems to have a complex and multifactorial pathogenesis (causes such as vasoactive substances in the CSF and the vessel wall, impairment of vasodilating activity, endothelial damage, immunoreactive or inflammatory processes). Endothelial injury and intimal platelet accumulation are the earliest arterial wall changes observed after SAH. A sudden rise in intracranial pressure, but not the presence of subarachnoid blood or acute arterial hypertension, is responsible for acute blood–arterial wall barrier disruption after SAH. In addition, in patients with aneurysmal SAH there seems to be a very significant independent...
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association between duration of initial loss of consciousness and delayed cerebral ischemia.13 Therefore, initial endothelial damage may be very important in causation of delayed vasospasm. This may be one significant reason why prevention and reversal of vasospasm have been so difficult, even during the early phase after SAH.3

In addition, there may be at least two phases of vasospasm, possibly occurring in an overlapping fashion.9,15,57 In the early (active) phase of vasospasm (<5–7 days post-SAH), vessel constriction can be partly reversed by vasodilators such as papaverine, but during the late (passive) phase (7–14 days post-SAH), pharmacological reversal of vessel constriction is difficult to achieve.

Endothelin-1 Synthesis and Function

There exist three structurally and pharmacologically separate ET isopeptides (ET-1, ET-2, and ET-3), of which only ET-1 is produced by endothelial cells. Endothelin-1 is also produced by neurons and astrocytes in the central nervous system.2,24 The prepro–ET-1 protein is 203 amino acids long and is processed to the 38- or 39-amino acid prohormone big ET-1, which is secreted by endothelial cells and circulates in plasma.3 Endothelin-1 is produced via proteolytic cleavage from big ET-1 by the ECE. The ET-1 is not stored in secretory granules within endothelial cells; instead, within minutes important stimuli such as hypoxia, ischemia, or shear stress induce the transcription of ET-1 mRNA and the synthesis and secretion of ET-1.32 The half-life of the mRNA is approximately 15 to 20 minutes, and the plasma half-life of ET-1 is approximately 4 to 7 minutes.25 Endothelin elimination from the bloodstream proceeds rapidly through the lungs, kidneys, and liver. Plasma ET-1 is 80 to 90% cleared by the lungs during the first passage. As much as 75% of ET-1 secretion from cultured endothelial cells proceeds toward the vasculature–smooth muscle interface than in the bloodstream.2,32 Plasma ET-1 measurements are, nevertheless, useful because plasma concentrations have been found to correlate well with the severity of vascular diseases.32

Endothelin-1 is the most powerful vasoconstrictor and inducer of long-lasting hypertension thus far discovered, with a potency 10 times that of angiotensin II.32,59,63 Extracellular calcium long and is processed to the 38- or 39-amino acid prepro-ET-1 protein, specifically big ET-1, which is secreted by endothelial cells; instead, within minutes important stimuli such as hypoxia, ischemia, or shear stress induce the transcription of ET-1 mRNA and the synthesis and secretion of ET-1.32 The half-life of the mRNA is approximately 15 to 20 minutes, and the plasma half-life of ET-1 is approximately 4 to 7 minutes.25 Endothelin elimination from the bloodstream proceeds rapidly through the lungs, kidneys, and liver. Plasma ET-1 is 80 to 90% cleared by the lungs during the first passage. As much as 75% of ET-1 secretion from cultured endothelial cells proceeds toward the vasculature–smooth muscle interface than in the bloodstream.2,32,35,52,59,65 In addition, ET-1 may also stimulate its own synthesis through ETB1 receptor activation in the endothelial cells.67 This may be very important in the presence of SAH, in which endothelin-derived relaxation seems to be impaired.35,52,59

Endothelin-1 is the most powerful vasoconstrictor and inducer of long-lasting hypertension thus far discovered, with a potency 10 times that of angiotensin II.32,59,63,65 Endothelin-1 causes prolonged, powerful arterial constriction.27 Most of the ET would be es calcium from intracellular calcium stores.32,35,52,59,65 Endothelin-1 is the most powerful vasoconstrictor and inducer of long-lasting hypertension thus far discovered, with a potency 10 times that of angiotensin II.32,59,63,65 Endothelin-1 may also stimulate its own synthesis through ETB1 receptor activation in the endothelial cells.67 This may be very important in the presence of SAH, in which endothelin-derived relaxation seems to be impaired.35,52,59

The effect of ET-1 is counteracted by EDRF (or NO) and prostacyclin.32,52,59,65 Endothelin-1 can also cause an increase in prostacyclin production, which inhibits platelet aggregation by an increase in platelet CAMP. This may also be one reason for the earlier observation14 of reduced platelet aggregation associated with hypodense release within the first few days after SAH, because plasma ET-1 levels were highest soon after SAH in this and another previous study.24 In the endothelium, ET-1 leads to activation of EDRF synthesis through ETB receptors, which causes relaxation of SMCs.32,52,59,65 Extracellular calcium is essential for ET activity, which cannot be inhibited by the dihydroxyproline class of calcium channel antagonists.32,52,59,65 Endothelin-1 binds to specific membrane receptors (ETα and ETβ in SMCs and ETβ in endothelial cells), leading to intracellular biochemical signals that involve the activation of phospholipase C, which hydrolyzes phosphatidylinositol-4,5-diphosphate to DAG, an activator of PKC, and inositol-1,4,5-triphosphate, which releases calcium from intracellular calcium stores.32,52,59,65

Both intraluminal and extraluminal ET have displayed potent and dose-dependent vasoconstricting effects on basilar arteries.57 The effects of intraluminal (but not extraluminal) ET was enhanced by extraluminal oxyHb and NG-monomethyl-L-arginine, an inhibitor of endothelial NO syn-

### TABLE 4

<table>
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<tr>
<th>Factor</th>
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<th>Phase III</th>
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<td>2.4 ± 0.5$</td>
<td>2.3 ± 0.5†</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>3.1 ± 0.8$</td>
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<td>2.7 ± 0.6</td>
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<tr>
<td>severe</td>
<td>3.2 ± 0.8§</td>
<td>2.7 ± 0.5§</td>
<td>2.3 ± 0.5</td>
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</table>

Values are expressed as the means ± SEM. See Tables 1 and 2 for classifications of phases and hypodense area groups. Vasospasm was estimated on the basis of postoperative angiograms obtained within 5 to 14 days post-SAH.

*p = 0.04, values were higher in Phase I than in Phase II. p = 0.048, values decreased by time from Phase I to Phase III. There were no significant interactions between time after SAH and grouping variables.

† p = 0.046 for Groups 2 and 3 compared with others in Phases I and II according to repeated-measures ANOVA; p = 0.098 between groups after adjustment for hypertension.

‡ p = 0.044 for difference between Group 3 and no hypodense area in Phases I and II according to repeated-measures ANOVA; p = 0.077 between groups after adjustment for hypertension.

§ p = 0.0347 for difference between severe vasospasm compared with other vasospasm groups in Phases I and II according to repeated-measures ANOVA; p = 0.067 between groups after adjustment for hypertension.

** p = 0.0519 for difference between diffuse vasospasm compared with none or localized vasospasm in Phases I and II according to repeated-measures ANOVA; p = 0.0426 between groups after adjustment for hypertension.

| Place of ET immunoreactivity to hypodense lesions on follow-up CT scans and angiographic evidence of vasospasm by elapsed time after SAH* |
|-------------------------------------------------|----------|
| Factor                                          | Phase I  |
| hypodense area                                  | Phase II |
| none                                            | Phase III|

Values are expressed as the means ± SEM. See Tables 1 and 2 for classifications of phases and hypodense area groups. Vasospasm was estimated on the basis of postoperative angiograms obtained within 5 to 14 days post-SAH.

*p = 0.04, values were higher in Phase I than in Phase II. p = 0.048, values decreased by time from Phase I to Phase III. There were no significant interactions between time after SAH and grouping variables.

† p = 0.046 for Groups 2 and 3 compared with others in Phases I and II according to repeated-measures ANOVA; p = 0.098 between groups after adjustment for hypertension.

‡ p = 0.044 for difference between Group 3 and no hypodense area in Phases I and II according to repeated-measures ANOVA; p = 0.077 between groups after adjustment for hypertension.

§ p = 0.0347 for difference between severe vasospasm compared with other vasospasm groups in Phases I and II according to repeated-measures ANOVA; p = 0.067 between groups after adjustment for hypertension.

** p = 0.0519 for difference between diffuse vasospasm compared with none or localized vasospasm in Phases I and II according to repeated-measures ANOVA; p = 0.0426 between groups after adjustment for hypertension.

The effect of ET-1 is counteracted by EDRF (or NO) and prostacyclin.32,52,59,65 Endothelin-1 can also cause an increase in prostacyclin production, which inhibits platelet aggregation by an increase in platelet CAMP. This may also be one reason for the earlier observation14 of reduced platelet aggregation and associated platelet thromboxane release within the first few days after SAH, because plasma ET-1 levels were highest soon after SAH in this and another previous study.24 In the endothelium, ET-1 leads to activation of EDRF synthesis through ETα receptors, which causes relaxation of SMCs.32,52,59,65 Extracellular calcium is essential for ET activity, which cannot be inhibited by the dihydroxyproline class of calcium channel antagonists.32,52,59,65 Endothelin-1 binds to specific membrane receptors (ETα and ETβ in SMCs and ETβ in endothelial cells), leading to intracellular biochemical signals that involve the activation of phospholipase C, which hydrolyzes phosphatidylinositol-4,5-diphosphate to DAG, an activator of PKC, and inositol-1,4,5-triphosphate, which releases calcium from intracellular calcium stores.32,52,59,65

Both intraluminal and extraluminal ET have displayed potent and dose-dependent vasoconstricting effects on basilar arteries.57 The effects of intraluminal (but not extraluminal) ET was enhanced by extraluminal oxyHb and NG-monomethyl-L-arginine, an inhibitor of endothelial NO syn-

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Endothelin secretion is also stimulated by cytokines and growth factors, and the mitogenic properties of ET can be used to argue for its possible role in remodeling and repair after vascular injury. Thrombin and oxyHb, which are present in SAH, can increase production and release of ET-1; ET receptor antagonists have been shown to reduce experimental vasospasm; thromboxane B2, the stable metabolite of thromboxane A2, from guinea pig alveolar macrophages, indicating also that ET-1 activates release of metabolites of arachidonic acid through phospholipase A2.

Endothelin-1 and Vasospasm After SAH

Are the ETs either mediators or markers of post-SAH vasospasm? Factors favoring the mediator theory include the following: 1) ET-1 and also big ET-1 after enzymatic conversion to ET-1 are extremely potent constrictors of cerebral vessels; 2) ET-1 causes histological changes that resemble post-SAH degenerative changes in the artery wall as well as chronic vasospasm, which is difficult to wash out; 3) ET receptor antagonists have been shown to reduce experimental vasospasm; 4) contractions caused by catecholamines, which are found in high concentrations in plasma and CSF after SAH and surgery (especially in patients with delayed cerebral ischemia and/or poor outcomes), are potentiated by threshold concentrations (3 × 10^-10 to 10^-9 M) of ET-1 in human arteries; 5) thrombin and oxyHb, which are present in high concentrations in CSF and plasma after SAH, can increase production and release of ET-1; and 6) increased levels of ET-1 occur in the plasma or CSF of patients with delayed cerebral ischemia.

Contradictory reports exist concerning plasma and CSF ET levels in cases in which there are delayed ischemic complications and vasospasm after SAH. In some studies, plasma ET-1, CSF ET-1, or big ET-1 concentrations have been correlated with vasospasm or have been found to increase after SAH. Contradictory to these results, plasma ET-1, CSF ET-1, and plasma big ET-1 concentrations have not been correlated with vasospasm. An increase in CSF ET-1 levels may be the result of cerebral ischemia rather than the cause of vasospasm. Although ET released from ischemic brain tissue does not increase plasma ET-1 levels, the release of ET-1 into CSF may cause a worsening of vasospasm because it is known to be difficult to reverse.

Suzuki, et al., demonstrated elevated plasma ET-1 levels in patients with SAH compared with levels in a control group. The values were highest soon after SAH and followed a gradual decline with increased time after SAH; the greatest values were among patients with symptomatic and/or angiographically observed vasospasm. On the other hand, another report the same research group demonstrated the highest ET-1 values on the 7th post-SAH day, paralleling the course of vasospasm. In both of these studies, plasma ET values among patients who had experienced SAH were clearly higher than those among controls. Although plasma ET-1 levels were elevated during the 1st week after SAH, especially in patients with vasospasm, CSF levels of ET-1 in those patients mainly increased during the 2nd week after SAH. The fact that the time course for elevated CSF ET-1 concentrations coincides with the occurrence of vasospasm suggests that ET-1 may also be an epiphenomenon of vasospasm without causality because during cerebral ischemia, ET-1 is released into CSF from astrocytes.

Postoperative relative concentrations of CSF ET-1 and big ET-1 were correlated to post-SAH vasospasm (increased blood flow velocity rate according to transcranial Doppler ultrasound), as well as to patient age. However, increases in transcranial Doppler ultrasound flow velocities in major basal arteries do not necessarily correlate very well with angiographically observed or symptomatic vasospasm. The initial postoperative CSF concentrations of big ET-1, ET-1, and ET-3 were significantly lower in patients with vasospasm than in other patients, but preoperative CSF levels of ET-1 and ET-3 were higher in those patients in whom a large amount of subarachnoid blood was observed on the CT scan. The authors explained this discrepancy by noting that all CSF ET concentrations directly correlated with patient age, with post-SAH vasospasm being significantly more common in younger patients. Preoperative plasma ET-1 and big ET-1 values, as well as the plasma big ET-1/ET-1 ratio, were significantly higher in patients who had experienced SAH than in healthy volunteers; however, they did not correlate with vasospasm nor were postoperative plasma ET levels thus correlated. In addition, preoperative ET-1 levels in patients who had or had not experienced SAH were similar.

In the present study, the prevalence of delayed ischemia and angiographically confirmed vasospasm was similar to those usually reported. Variables related to delayed ischemia (symptomatic vasospasm or delayed ischemic deterioration, angiographic evidence of vasospasm, and post-SAH cerebral infarction) were analyzed separately to determine how ET-1 levels could possibly correlate with the pathophysiological characteristics of delayed ischemia. In this instance, variables were not combined to place more patients in the ischemia group, as was done in previous studies. Plasma and CSF ET concentrations have differed significantly between studies in which the prevalence of vasospasm has, for some reason, usually been greater than expected. Conflicting results suggest that ET-1 plays no significant role in vasospasm.

Endothelin concentrations in the present study were obtained using a commercially available enzyme immunoassay kit, and were similar to those of healthy volunteers except in those patients in whom there were ischemic symptoms, vasospasm-related cerebral infarctions, or angiographic evidence of severe or diffuse vasospasm. Recently, our research group observed similar ET-1 levels in a series of 51 patients who had experienced SAH and a control group; the levels of ET-1 were determined using...
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radioimmunoassay techniques (unpublished data). The significance of the results of the present study may be somewhat underestimated because the crossreactivity for ET-3 was 66% and plasma ET-3 levels have not been shown to be changed by the occurrence of SAH. In addition, ET-1 levels in hypertensive patients documented in previous reports are similar to those obtained in the present study. However, ET-1 levels were lower in patients with hypertension than in those with post-SAH vasospasm. In this study, ET concentrations did not correlate with the amount of blood in the subarachnoid space. This was most likely because Fisher’s classification did not provide an accurate estimate of blood amount; the majority of patients had a thick layer of blood or clots in the vertical cisterns.

Although ET levels that could be measured in peripheral venous blood were low, they likely reflected the production of ET-1 in the cerebral arteries because the patients had no symptomatic arterial injury or vasculopathy in their noncerebral vasculature, a finding that could explain differences in ET-1 levels between patients with delayed ischemia and those without ischemia; however, a history of hypertension somewhat decreased the association in these patients between ischemic complications and ET levels. In addition, ET-1 levels decreased in patients with ischemia as time increased after SAH, indicating that SAH and an associated delayed ischemia were responsible for these elevated values.

Endothelin concentrations invariably were found to be increased in the endothelium or vessel wall of a vasospastic artery 2 to 3 days after experimental SAH but not on the 7th day. This finding agrees well with that of the present study, and the plasma ET-1 level may reflect the concentration in cerebral vessels, especially because ET-1 levels in the present study correlated with diffuse vasospasm. Expression of ET\textsubscript{A} receptor mRNA has also been found to be markedly higher on the 3rd day after experimental SAH and detected to a lesser extent on the 7th day. In another study, levels of ET\textsubscript{A} receptor mRNA in arteries and levels of ET\textsubscript{A} and ET\textsubscript{B} receptor mRNAs in the cortex were higher 7 days after SAH, whereas levels of ET\textsubscript{B} receptor, prepro–ET\textsubscript{1}, and prepro–ET\textsubscript{3} mRNAs in cerebral arteries, and levels of prepro–ET\textsubscript{1} and prepro–ET\textsubscript{3} in the cortex were not. In the study by Hino, et al., the authors suggested that changes in the cerebral cortex after SAH might be secondary to ischemia. The levels of ET\textsubscript{A} receptors they measured were much lower in cerebral arteries than the levels of ET\textsubscript{B} receptors. However, in one study, immune reactive ET-1, big ET-1, and ECE concentrations in the artery wall were still increased on the 7th post-SAHA day.

Most experimental studies show that vasospasm may be reduced or prevented by inhibitors of ECE (intracisternal phosphoramidon\textsuperscript{47} or intravenous and topical administration of CGS 26303\textsuperscript{39,46}) by intracisternal,\textsuperscript{12,17,20,40} oral,\textsuperscript{68} or intravenous\textsuperscript{58} administration of ET\textsubscript{A} receptor antagonists, by intracisternal or topical administration of ET\textsubscript{B} and ET\textsubscript{B1} receptor antagonists,\textsuperscript{52} by systemic or oral post-SAH administration of bosentan (a nonpeptidic ET\textsubscript{A} and ET\textsubscript{B} receptor antagonist),\textsuperscript{46,47,60} and also more nonspecifically by actinomycin D (an inhibitor of mRNA synthesis).\textsuperscript{49,50} Bosentan, in addition to its attenuating effect on experimental vasospasm\textsuperscript{48,66} and essential hypertension in humans,\textsuperscript{59} also increased plasma but not CSF levels of ET-1. In two studies,\textsuperscript{49,68} daily intracisternal administration of the ECE inhibitor phosphoramidon and the ET\textsubscript{A} receptor antagonist BQ-123\textsuperscript{49} produced no significant reduction in experimental vasospasm.

In most of these experimental studies a pretreatment protocol was included (that is, medicine was given before or at the time of experimental SAH) or the effect on short-term vasospasm (2–3 days after SAH) was studied, neither of which is relevant pathophysiologically or clinically to delayed post-SAH vasospasm in humans.\textsuperscript{35,59} The inhibitor of ECE, CGS 26303, attenuates significantly less vasospasm when administered after experimental SAH than when given before SAH.\textsuperscript{4} The beneficial effect of CGS 26303 therapy decreases quite quickly within 24 hours after SAH.\textsuperscript{35,59} The same situation occurred in the intravenous administration of the ET\textsubscript{A} receptor antagonist TBC 11251.\textsuperscript{48} In addition, several potentially effective treatments, including intrathecal thrombolysis, have been shown to be beneficial in experimental vasospasm studies, but almost all of these treatments have proved useless in the prevention or reversal of ischemic symptoms, which are due to large artery vasospasm after aneurysmal SAH in humans.\textsuperscript{35,59}

An inability to reverse completely ET-1–induced constriction by an ET antagonist suggests the following possibilities: 1) both the ET\textsubscript{A} and ET\textsubscript{B} receptors in vascular smooth muscle may be involved; 2) an established vasospasm may be only partially reversible; and 3) other events may be involved, such as vessel injury induced by free radicals, direct vasoconstriction by oxyHb, or inhibition of EDRFs.

Mechanism of ET-1–Induced Post-SAH Vasospasm

Because EDRF activity in endothelial cells is diminished after SAH, there may be a localized ET-1/EDRF imbalance in the cerebral arteries after hemorrhage.\textsuperscript{45,52,59} This functional imbalance can favor ET-induced vasoconstriction, even without any marked ET production. The finding of the present study that plasma ET-1 levels correlated better with larger hemispheric infarctions than with central lacunar infarctions agrees with the concept that, after SAH, NO-mediated dilation is not impaired (or cannot be detected) in small perforating arterioles, but is impaired in large cerebral arteries.\textsuperscript{52}

After SAH, ET-1 release from endothelial cells can be stimulated by thrombin, epinephrine, TGF\textbeta, angiotensin II, vasopressin, oxyHb, cytokines, or platelets.\textsuperscript{2,5,35,39,63,65} In an experimental model of vasospasm, expression of TGF\textbeta, which is also an important regulator of collagen synthesis, displayed a marked increase in an artery 3 days after the artery was exposed to a periarterial blood clot and then gradually declined.\textsuperscript{25}

Oxyhemoglobin produces a concentration–time relationship (0.1–10 μM) and time–(0–24 hours) dependent increase in ET-1 production from both vascular SMCs and endothelial cells.\textsuperscript{26,41} This stimulation is more prominent than that caused by thrombin or phorbol 12-myristate 13-acetate (a PKC activator), but this rate was 30-fold lower in SMCs than in endothelial cells, in which oxyHb also caused ET-1 mRNA induction for 1.5 to 6.5 hours. Protein kinase C inhibition by staurosporine reduced this oxyHb-induced
ET-1 production in both muscle and endothelial cells, but it was inhibited by cAMP only in SMCs.\textsuperscript{49} In addition to directly stimulating basal production of immunoreactive ET-1, oxyHb significantly augmented immunoreactive ET-1 production following platelet-mediated stimulation of ET production.\textsuperscript{41} Oxyhemoglobin-induced ET-1 production in endothelial cells was regulated by PKC, and in SMCs by both PKC and the cAMP-dependent pathway. On the other hand, oxyHb did not seem to affect ET-1 production through either a cGMP pathway or phospholipase C. On the other hand, Pluta et al.\textsuperscript{45} could show no relationship between oxyHb and ET-1 production in astrocytic or endothelial cell cultures.

Both \textit{L}-N\textsuperscript{6}-monomethyl arginine and methylene blue (a soluble guanulate cyclase inhibitor) potentiate thrombin-induced ET-1 production, probably via cGMP pathways, and the production was reduced by superoxide dismutase (a scavenger of superoxide anions that inactivate ET-de-\textit{derived NO}) and 8-bromo-cGMP (a nonhydrolyzable analog of cGMP), whereas the basal ET-1 release was unaffected.\textsuperscript{26,52,59} On the other hand, inhibition of cGMP by N\textit{N}\textsuperscript{nitro-L-arginine methyl ester hydrochloride (an NO synthase inhibitor) and by methylene blue did not potentiate oxyHb-induced ET-1 production, which was, however, reduced by superoxide dismutase and 8-bromo-cGMP.\textsuperscript{26} Oxyhemoglobin inhibits endothelium-dependent relaxation by binding endothelium-derived NO in the extracellular space before it is able to diffuse into the SMCs. In the study by Kasuya et al.,\textsuperscript{26} the authors suggest that the oxyHb stimulates immunoreactive ET production rather than inhibits a cGMP pathway.

Actinomycin D may prevent experimental vasospasm better than ET antagonists.\textsuperscript{49,50} Interference with multiple biochemical pathways may explain its protective effect: actinomycin D may inhibit induction of several peptic vasoactive factors within the spastic lesion.\textsuperscript{50} Candidates for these signaling molecules may include the following: 1) cell growth factors such as PDGF and TGF\textit{B}, 2) vasoconstrictors such as ET, and 3) certain groups of cellular protooncogenes such as \textit{c-myc}, \textit{c-fos}, and \textit{c-jun}, which are involved in nuclear signal transduction events in response to extracellular stimuli.\textsuperscript{50} Many of these factors, such as ET and PDGF, exhibit both mitogenic and vasocostricting activities. Furthermore, in the presence of SAH, mutual induction of these signaling molecules may occur. Substances derived from blood clots and platelets such as thrombin, TGF\textit{B}, and oxyHb can potently induce ET mRNA,\textsuperscript{26,61} and ET, in turn, can induce cellular protooncogenes such as \textit{c-myc} and \textit{c-fos}.\textsuperscript{7} The effect of actinomycin D in the treatment of vasospasm may also be nonspecific, occurring via suppression of inflammation and immunological reactions as well as via other responses that require RNA synthesis.\textsuperscript{50}

Endothelin-1 can cause vasospasm by increasing intracellular DAG levels. Endothelin-1 and, to a lesser extent, erythrocyte hemolysate, produced sustained SMC contraction that was associated with increased DAG, whereas agonists such as K\textsuperscript{+}, angiotensin II, vasopressin, or PDGF failed to increase DAG levels.\textsuperscript{61} Diacylglycerol is an endogenous PKC activator and is probably a mediator of chronic vasospasm because PKC mediates sustained and possibly partially calcium-insensitive SMC contraction.\textsuperscript{61} In addition, PKC may mediate fibroblast-induced contrac-
tion of the extracellular matrix, which is relatively resistant to vasodilating agents.\textsuperscript{32}

Endothelin may also induce liberation of arachidonic acid metabolites (vasoconstrictive prostaglandins from the endothelium and thromboxane from platelets) through activation of phospholipase A\textsubscript{2}.\textsuperscript{36,56} Platelet thromboxane release may be important in development of vasospasm during the 1st week after aneurysmal SAH.\textsuperscript{22,24}

\textbf{Hypertension, Cigarette Smoking, and Vasospasm}

Hypertension has been shown to increase, independently from other factors, the risk of cerebral infarction after SAH.\textsuperscript{42} Cigarette smoking also has been shown, in a large metaanalysis, to increase the risk of symptomatic vasospasm.\textsuperscript{58} A history of hypertension and cigarette smoking were both associated significantly in this study with the occurrence of post-SAH cerebral infarction, and hypertension also correlated with symptomatic vasospasm. Cigarette smoking was associated almost significantly with symptomatic vasospasm; this may be in part due to chance in this study because of the relatively small patient population compared with that of a metaanalysis.\textsuperscript{60}

In the present study, no clear association could be shown among hypertension, smoking, and angiographically demonstrated vasospasm in large cerebral arteries during the 2nd week after SAH. Thus, a history of hypertension and of smoking must depend on mechanisms of action other than vasospasm of large arteries, by which they would increase the risk of infarction and symptomatic vasospasm after SAH. By impairing the collateral circulation, ET may be one mechanism by which hypertension increases risk for delayed ischemia after SAH; however, this effect cannot explain the association between smoking and increased risk for cerebral infarction after SAH.

Recently, bosentan has been shown to reduce blood pressure values in patients with essential hypertension.\textsuperscript{29} It would also be interesting to know whether bosentan or other ET antagonists can reduce the severity and distribution of angiographic vasospasm as well as delayed ischemia after aneurysmal SAH in humans. However, a patient receiving bosentan may need more intensive hypervolemia–hypertension treatment because bosentan may reduce blood-pressure values and, thereby, increase ischemic symptoms if angiographically confirmed vasospasm is not attenuated by this drug.

\textbf{Conclusions}

The results of this study show that plasma ET concentrations are elevated in patients with delayed cerebral ischemia and FND, angiographic evidence of severe or diffuse vasospasm, and hypodense lesions caused by delayed ischemia within the first 2 weeks, especially the first 5 days, after aneurysmal SAH. Otherwise, ET concentrations were similar to those of healthy volunteers. This increased ET immunoreactivity may be a consequence of endothelial dysfunction or damage to cerebral arteries and an indication of a repair process, and/or it may be a result of increased ET-1 release from endothelial cells due to various inducers such as thrombin, catecholamines, growth factors, or oxyHb. In addition, a history of hyper-
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tension and of smoking before SAH seem to increase the frequency with which hypodense lesions due to delayed cerebral ischemia appear on follow-up CT scans and also the frequency of symptomatic, but not angiographically confirmed, vasospasm. Thus, a history of hypertension and of smoking may worsen symptoms due to vasospasm. Endothelin-1 may mediate this worsening caused by hypertension, but the mechanism by which smoking impairs ischemia remains unknown.

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