Endothelin-1 in Subarachnoid Hemorrhage
An Acute-Phase Reactant Produced by Cerebrospinal Fluid Leukocytes

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Background and Purpose—The most potent vasoconstrictor known, endothelin-1, is currently considered to mediate cerebral vasospasm in subarachnoid hemorrhage (SAH), which can cause delayed cerebral ischemia. In our study, we performed clinical and in vitro experiments to investigate the origin and the mechanisms of the secretion of endothelin-1 in SAH.

Methods—Endothelin-1 and markers of inflammatory host response (interleukin [IL]-1β, IL-6, and tumor necrosis factor-α) were comparatively quantified in the cerebrospinal fluid (CSF) of SAH patients and control subjects, and concentrations were related to clinical characteristics. Furthermore, mononuclear leukocytes isolated from the CSF of SAH patients and control subjects were analyzed regarding their mRNA expression of endothelin-1 and inflammatory cytokines. Finally, complementary in vitro experiments were performed to investigate whether coincubation of blood and CSF can trigger leukocytic mRNA expression and release of these factors.

Results—Activated mononuclear leukocytes in the CSF of SAH patients synthesize and release endothelin-1 in parallel with known acute-phase reactants (IL-1β, IL-6, and tumor necrosis factor-α). Complementary in vitro experiments not only further confirmed this leukocytic origin of endothelin-1 but also showed that aging and subsequent hemolysis of blood is sufficient to induce such endothelin-1 production.

Conclusions—The demonstration that endothelin-1 is produced by activated CSF mononuclear leukocytes suggests that subarachnoid inflammation may represent a therapeutic target to prevent vasospasm and delayed cerebral ischemia after SAH. (Stroke. 2000;31:2971-2975.)

Key Words: cerebral ischemia ■ cytokines ■ endothelins ■ subarachnoid hemorrhage ■ vasospasm

Subarachnoid hemorrhage (SAH) most commonly occurs when an aneurysm in a basal cerebral artery ruptures. Among patients who survive this event, the major cause of death and disability is constriction of the large cerebral arteries, leading to delayed cerebral ischemia, the “second stroke.”

Several lines of evidence implicate endothelin-1 (ET-1), the most potent and persistent vasoconstrictor known to date, in the pathophysiology of cerebral vasospasm after SAH. First, levels of ET-1 are increased in the cerebrospinal fluid (CSF) and plasma of SAH patients in close correlation with development of vasospasm. Second, delayed vasospasm can be experimentally evoked by the administration of ET-1. Third, antagonists of ET-1 attenuate vasospasm in experimental models of SAH. However, mechanisms of release, cellular origin, or even the compartment of release of ET-1 in SAH are still unknown. So far, production of ET-1 has been attributed to endothelial cells, smooth muscle cells, neurons, or astrocytes.

Earlier studies described inflammatory changes in SAH, ie, subarachnoid and perivascular leukocytic infiltrates, or increased CSF concentrations of inflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α in relation to the development of vasospasms. These reports, together with the recent observation that constrictions of the basal cerebral arteries occur also in bacterial meningitis, argue for a role of inflammation in the pathogenesis of cerebrovascular complications in SAH.

In the present study, we performed complementary clinical and in vitro investigations to test the hypothesis that the vasoconstrictor ET-1 could be synthesized and released by CSF leukocytes as part of a subarachnoid inflammatory host response triggered by SAH.

Subjects and Methods

Patients and Control Subjects
In total, 35 patients (21 females and 14 males), aged 23 to 76 (median 56) years, with SAH caused by aneurysmal rupture were studied. Hunt and Hess scores to classify disease severity were 1 in 9%, 2 in 11%, 3 in 32%, 4 in 29%, and 5 in 18% of the patients.
Except for 4 patients, all patients underwent surgical obliteration of the aneurysm <72 hours after the onset of symptoms. Therapy consisted of combined induction of hypervolemia, hypertension, and hemodilution (triple H therapy) alone in 49%, triple H therapy combined with nimodipine in 20% or angiographically administered papaverine in 6%, nimodipine alone in 6%, and no therapy/other therapy in 19% of the patients.

Patients had serial analyses of transcranial Doppler sonography to record abnormalities of systolic cerebral blood flow velocity (CBFV) transtemporally in the proximal segments of the major basal cerebral arteries by use of a DWL Multipod X TCD device (DWL Sipplingen) exactly as described previously.24 All patients had follow-up CT at least twice to assess possible delayed cerebral ischemia. In contrast, follow-up angiography was performed in only some of the patients (when clinically indicated).

Furthermore, we included 20 control subjects (12 females and 8 males), aged 23 to 80 (median 40) years. These had lumbar puncture performed for the exclusion of initially suspected meningitis or hemorrhage, which could be subsequently excluded by clinical, neuroradiological, and laboratory workup, including CSF analysis. The present study was approved by the ethical committee of the Mannheim Clinic, University of Heidelberg.

**Analysis of CSF Concentrations and Leukocytic Synthesis of ET-1 and Inflammatory Mediators in SAH**

At day 5 after the onset of the first symptoms, CSF for analysis of release of ET-1 and cytokines was obtained in all 35 patients either by ventricular drainage (n = 26) or by lumbar puncture (n = 9). In addition, in 13 of these SAH patients and in 10 of the control subjects, mononuclear leukocytes were isolated within 72 hours after the onset of symptoms and again at day 5 for further polymerase chain reaction (PCR) analyses of leukocytic mRNA expression of ET-1, IL-1β, IL-6, and TNF-α.

**Analysis of Release and Leukocytic Synthesis of ET-1 and Inflammatory Mediators in In Vitro–Coincubated Blood and CSF**

To reproduce the presumed conditions in the subarachnoid space of patients with SAH, CSF and autologous blood from 10 of the patients (when clinically indicated) were used for PCR analysis. RNA was obtained from the isolated cells with the use of acid phenol and stored at −80°C until used. To test for the timing and conditions of possible ET-1 release, coincubation experiments were isolated by a standard density gradient centrifugation with the use of Ficoll (Biochrom).25 Total cellular RNA was obtained from the isolated cells with the use of acid phenol extraction as previously described.26 Oligo(dt) (12–18– primed reverse transcription was performed with the use of 1 μg of total RNA in 20 μL of reaction volume.

PCR for ET-1 was performed as duplex PCR with GAPDH as the housekeeping gene. The following primers were used: ET-1 forward primer 5’-AGTGCATCCTCGAATGTGACATAA-3’ and reverse primer 5’-TCTTCACCATTCGTTGCTTGG-3’, corresponding to base pairs 5684 to 5703 and 7270 to 7251 of the human ET-1 gene (GenBank No. J05008, product size 158 bp). GAPDH forward primer 5’-CTTCCACCAGGATGACAAGCA-3’ and reverse primer 5’-GGTTTTGCGGCCATCACGCCACAGTTT-3’ corresponding to base pairs 99 to 120 and 9519 to 9499 of the human GAPDH gene, respectively.27 PCR consistency was evaluated by agarose gel electrophoresis and sequencing of the PCR products.

**Statistical Analysis**

Results are expressed as mean±SEM. The Mann Whitney U test or the Fisher exact test, each with a Bonferroni correction, was used as appropriate. For correlation, the Pearson correlation coefficient was used. Statistical significance was considered at P <0.05.

**Figure 1.** Release of ET-1 and inflammatory cytokines in SAH patients (n=35) and control subjects (CON, n=20) and in CSF/blood coincubation experiments (in vitro, n=10). *P<0.05 (after Bonferroni correction) compared with normal CSF values.

CGTCTTCACCACATGGAGA-3’ and reverse primer 5’-CGGCCATCACGCCACAGTTT-3’, corresponding to base pairs 365 to 384 and 607 to 623 (GenBank No. M17701, product size 259 bp). Cycling conditions were 5 minutes at 95°C, 40 cycles of 45 seconds at 94°C, 45 seconds at 59°C, and 45 seconds at 73°C, followed by 7 minutes at 73°C. PCR for expression of IL-1β, IL-6, and TNF-α was carried out with primers from the commercially available GeneXpress kit (Biosource Europe). β-Actin was used as the housekeeping gene. The sequences of β-actin primers were identical in sequence, as recently described.27 PCR with inflammatory cytokine primers was performed as recommended by the manufacturer.

**Determination of Concentrations of ET-1 and Proinflammatory Cytokines**

ET-1 in the CSF of SAH patients and control subjects and in the CSF/blood coincubation experiments was extracted from acidified samples on C18 columns by adding acetic acid and was evaporated under nitrogen gas. After reconstitution in the assay buffer, the extracted ET-1 was measured by radioimmunoassay (Nichols Institute Diagnostics), as recently described.23 Concentrations of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were quantified with sandwich immunoassays (R&D Systems) exactly as described previously.

**Results**

CSF Mononuclear Leukocytes From SAH Patients Produce ET-1 in the Context of a Compartmentalized Inflammation

At a protein level, ET-1 was detectable in the CSF of 46% of the SAH patients but in none of the control subjects. Mean concentrations are shown in Figure 1. Also, CSF concentrations of IL-1β, IL-6, and TNF-α were significantly increased...
CSF concentrations of ET-1 were associated with those of proinflammatory cytokines; ie, concentrations of ET-1 correlated with levels of IL-1\(\beta\) (\(r=0.45, P<0.05\)), IL-6 (\(r=0.52, P<0.01\)), and TNF-\(\alpha\) (\(r=0.57, P<0.01\)).

Importantly, we showed that the mononuclear leukocytes of SAH patients are the source of ET-1 and inflammatory cytokines because they expressed significantly increased amounts of mRNA of these molecules (Figure 2, Table 1). Apart from CSF, we also detected ET-1 in the peripheral blood (2.92±1.88 pg/mL at day 5).

In Vitro Coincubation of CSF and Blood Triggers Leukocytic Release of ET-1 in Parallel With Release of Inflammatory Mediators

Coincubation of CSF and autologous blood from healthy subjects induced mononuclear leukocytes to synthesize ET-1 together with inflammatory mediators (IL-1\(\beta\), IL-6, and TNF-\(\alpha\)), as proven by the observation of increased concentrations of these proteins in the supernatant (Figure 1) and the detection of mRNA expression (Figure 2, Table 1). Release of ET-1 in vitro was detectable, with values in the range of those observed in SAH, and concentrations of inflammatory cytokines were also increased up to 100-fold (Figure 1). In separate experiments, we found that ET-1 protein increased in incubated blood or in coincubated blood/CSF within the first 12 hours (Table 2).

Relationship Between CSF Concentrations of ET-1 in SAH and Clinical Characteristics

We subdivided the patients into subpopulations with higher (≧210 cm/s) and lower (<210 cm/s) CBFV during the first 11 days according to cutoff values established in earlier work.\(^{24}\) Development of higher CBFV (n=22) was associated with significantly increased CSF concentrations of ET-1 compared with the presence of lower CBFV (n=13) (ie, 1.69±0.28 versus 0.47±0.18 pg/mL, respectively; \(P<0.01\)). Follow-up CT revealed signs for newly developed cerebral ischemic injury in 46% of the SAH patients. These patients exhibited a trend (although nonsignificant) toward higher CSF concentrations of ET-1 (1.41±0.29 versus 1.08±0.30 pg/mL). No significant correlation between ET-1 concentrations and Hunt and Hess scores or scores of the Glasgow outcome scale were found.

Discussion

ET-1 is currently intensively studied as a key factor in the pathogenesis of vasospasm after SAH.\(^{1}\) In the present study, we proved on an mRNA and a protein level that mononuclear leukocytes in the subarachnoid space of SAH patients are the producers of this vasoconstrictor. The parallel cosynthesis of ET-1 and inflammatory cytokines by mononuclear leukocytes together with the significant correlation between ET-1 and inflammatory mediators indicates that this vasoconstrictor is synthesized as an acute-phase reactant in the context of a subarachnoid inflammation. Interestingly, the role of acute-phase reactant has also been suggested in light of the detection of a consensus sequence for acute-phase elements in the human ET-1 gene.\(^{28}\)

Our complementary in vitro experiments not only confirm this leukocytic ET-1 release but demonstrate that aging of blood or blood/CSF (in the obvious absence of endothelial

**TABLE 1.** Percentages of Detectable mRNA of ET-1 and Inflammatory Cytokines in Mononuclear Leukocytes From CSF of Control Subjects (n=10) and SAH Patients (n=13) and in CSF/Blood Coincubation Experiments (n=10)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>mRNA, %</th>
<th>In Vitro Coincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Subjects</td>
<td>SAH Patients</td>
</tr>
<tr>
<td>ET-1</td>
<td>20</td>
<td>20 (92*)</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>10</td>
<td>10 (46)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>0 (23)</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>0</td>
<td>0 (62*)</td>
</tr>
</tbody>
</table>

*\(P<0.05\) (after Bonferroni correction).

**TABLE 2.** Temporal Profile of ET-1 Release in Blood Coincubated With or Without Autologous CSF (n=5)

<table>
<thead>
<tr>
<th>Incubation Interval</th>
<th>ET-1 Release, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood + CSF</td>
</tr>
<tr>
<td>0 h</td>
<td>0.45±0.19</td>
</tr>
<tr>
<td>3 h</td>
<td>0.67±0.26</td>
</tr>
<tr>
<td>12 h</td>
<td>7.23±3.31</td>
</tr>
<tr>
<td>24 h</td>
<td>3.32±2.61</td>
</tr>
</tbody>
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**Figure 2.** Reverse transcription–PCR showing expression of mRNA of ET-1 and of inflammatory cytokines in mononuclear leukocytes. GAPDH mRNA or actin was used as an internal standard. MW indicates molecular weight marker; ctl, CSF of control subjects; 1, CSF of SAH patients; and 2, in vitro CSF/blood coincubation experiments.
cells, neurons, astrocytes, or other discussed cellular sources of ET-1) is sufficient to trigger leukocytic synthesis of ET-1. Interestingly, the ability of leukocytes to produce ET-1 has been observed in earlier in vitro work.29–32

We also detected ET-1 in peripheral blood in the range of concentrations previously reported.9 Systemic stress response could explain the release of ET-1 in the systemic circulation. Because levels in CSF were similar or even more elevated than in peripheral blood in many patients, ET-1 detected in CSF was unlikely to be derived only from the systemic circulation.

The biological role of ET-1 release by CSF leukocytes is unclear. In many inflammatory conditions, vasoconstrictive effects of activated mononuclear leukocytes could represent a beneficial host defense mechanism, eg, a mechanism that limits the spreading of infectious agents or toxic mediators. In SAH, however, an uncontrolled leukocytic production of this vasoconstrictor could contribute to persistent vasospasm and subsequent cerebral ischemia.

Once released in the subarachnoid space, ET-1 (2.5 kDa) can easily access the smooth muscle cells of contiguous basal arteries from their adventitial side, because much larger molecules (eg, horseradish peroxidase, molecular mass 40 kDa) have been demonstrated to pass from the cisterna magna through the vessel wall to the basal membrane within minutes.33 This is possible because the surfaces of the pial cerebral arteries are, exceptionally, not confined by collagen or fibroblasts but are in direct contact with the nourishing CSF.33 Indeed, it has been shown that ET-1 acts from the adventitial but not the luminal side of the vessel.34 This is consistent with our observation of a release of ET-1 by leukocytes in the subarachnoid space. Interestingly, the leukocytic generation of ET-1 shown in the present study could also explain the recently reported vasospasms in bacterial meningitis,23 a disease that does not involve most of the cells, neurons, astrocytes, or other discussed cellular sources of ET-1.

Our observation that subjects with evidence of severe vasospasms (higher CBFV) exhibit higher ET-1 concentrations in CSF is in accordance with some5–9,35 but not all9 earlier studies that reported possible associations between ET-1 levels and cerebrovascular complications. It must be noted that ET-1 may also act in a paracrine fashion36; therefore, a strong correlation between ET-1 concentration and vasospasm is not mandatory to establish the pathogenetic importance of ET-1 in SAH.

In conclusion, the present study shows for the first time, to our knowledge, that ET-1 is subarachnoidly produced by CSF mononuclear leukocytes in the context of a compartmentalized inflammatory host response and that blood-CSF contact acts as a trigger for such leukocytic ET-1 synthesis. Therefore, we speculate that early subarachnoid administration of anti-inflammatory drugs (eg, during aneurysm surgery) could reduce local leukocytic ET-1 release and possibly prevent delayed cerebral ischemia after SAH.

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