Decreased Cerebrospinal Fluid Apolipoprotein E After Subarachnoid Hemorrhage
Correlation With Injury Severity and Clinical Outcome

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Background and Purpose—The apolipoprotein E (APOE) e4 allele has been associated with unfavorable outcome after subarachnoid hemorrhage (SAH), suggesting that apoE plays an important role in the response of the brain to SAH. We determined the concentration of apoE in the cerebrospinal fluid (CSF) of patients with SAH and a control group to test the hypothesis that alterations in CSF apoE reflect the response of the brain to SAH and are correlated with the severity of injury and outcome.

Methods—ApoE and S100B (a marker of brain injury) were measured by ELISA in CSF from a non–brain-injured control group and patients with SAH. The severity of SAH was determined from the Glasgow Coma Scale, and the clinical outcome was determined from the Glasgow Outcome Scale.

Results—in contrast to increased CSF concentration of S100B, CSF apoE concentration was significantly lower in patients after SAH than in control subjects (Mann-Whitney test, P<0.0001). SAH patients with more severe injury and less favorable outcome had lower CSF apoE concentration than did patients with milder injury and better clinical outcome (Fisher exact test, P=0.02).

Conclusions—the concentration of apoE in the CSF decreases after SAH, despite the likely leakage of plasma apoE into the CSF. We speculate that apoE is retained within the parenchyma of the central nervous system in response to injury, where, in view of previous data, it may have a protective role. (Stroke. 2003;34:637-642.)

Key Words: apolipoproteins ■ cerebrospinal fluid ■ outcome ■ subarachnoid hemorrhage

In the central nervous system (CNS), apolipoprotein E (apoE indicates protein; APOE, gene) functions as a key regulator of cholesterol and lipid transport and facilitates the delivery of cholesterol for synaptogenesis.1 In humans, the APOE gene is polymorphic (e3/3, e3/4, e4/4, e2/3, e2/4, and e2/2), and epidemiological studies identify the APOE e4 allele as a genetic risk factor for ischemic heart disease and Alzheimer’s disease.2,3 In addition, possession of APOE e4 has been associated with increased mortality (and a more severe outcome in survivors) after several different forms of acute brain injury, including injury due to subarachnoid hemorrhage (SAH), spontaneous intracerebral hemorrhage, cardiopulmonary bypass surgery, cardiopulmonary resuscitation, and trauma.4-8 Although these studies suggest a role for apoE in the response to human acute brain injury (a concept supported by animal models), the mechanism underlying these observations remains uncertain, and in vivo evidence from humans is lacking.9-14

We hypothesized that the role of apoE as an important factor in the response of the brain to SAH would be reflected by alterations in the concentration of apoE in the cerebrospinal fluid (CSF) and that these alterations may be correlated with the severity of the injury and with outcome. In addition to apoE, we assayed the mainly astrocyte-derived protein S100B to quantify the release of brain-specific proteins into the CSF after SAH, and we assayed the plasma protein albumin to quantify the release of plasma-derived proteins into the CSF after SAH.15

Subjects and Methods

Patients and Control Subjects
We conducted a prospective cohort observational study of patients with spontaneous SAH who required ventriculostomy for drainage of CSF and/or intraventricular pressure monitoring. The patients were admitted to the neurosurgical unit in Pittsburgh, Pa, between September 1998 and February 2001. Injury severity was stratified clinically by using the best recorded Glasgow Coma Scale (GCS)
CSF Sample Collection

The CSF from patients with SAH was sampled from the ventricular drainage system within 3 days of the hemorrhage. One CSF sample per patient was analyzed. CSF from the control subjects was sampled from the lumbar subarachnoid space. The CSF samples were processed in an identical manner: they were rapidly frozen after sampling in polypropylene collection vials and stored at −80°C until they were analyzed within 3 months of freezing. Before analysis, the CSF underwent 1 freeze-thaw cycle, and once it was thawed, a protease inhibitor cocktail (product No. P 2714, Sigma Chemical Co) was added to the CSF (final concentration 1 mmol/L).

ApoE Assay

The concentration of apoE in CSF was determined as previously described.19 In brief, rabbit polyclonal anti-human apoE antibody (Dako) diluted in 0.02 mol/L citrate buffer was used as the capture antibody. The coated plate was washed with PBS and then blocked with 2% BSA. The blocked plate was then washed with PBS/Tween before incubation at 37°C with diluted CSF samples, and the apoE standard curve (1.5 to 100 μg/L) was measured in duplicate. After further washing with PBS/Tween, goat anti-human apoE antibody (Chemicon) was used as the detection antibody, followed by horseradish peroxidase–conjugated rabbit anti-goat IgG as the secondary antibody for the color reaction as described above. There was parallelism between the apoE calibration curve and serial dilutions of control CSF, SAH CSF, and mixtures of plasma and control CSF or lysed red blood cells and control CSF. The recovery of apoE added to these preparations ranged from 95% to 98%. The intra-assay and interassay coefficients of variation were 7.4% and 8.6%, respectively, and the lower limit of detection was 3 μg/L.

S100B Assay

The concentration of S100B in the CSF was assayed as previously described.20 In brief, 96-well microtiter plates were coated with 200 μL of 0.05 mol/L carbonate buffer containing monoclonal anti-S100B (Affinity Research Products). The plates were washed with 0.67 mol/L barbitone buffer containing 5 mmol/L calcium lactate, 0.1% BSA, and 0.05% Tween and then blocked with 2% BSA and washed again. CSF samples diluted in 0.67 mol/L barbitone buffer containing 5 mmol/L calcium lactate were added in duplicate. After incubation and washing, horseradish peroxidase–conjugated polyclonal anti-S100B (Dako) was used as the detecting antibody. The σ-phenylenediamine color reaction was stopped with 1 mol/L hydrochloric acid, and the absorbance was read at 492 and 405 nm. The antigen concentration was calculated from an internal standard curve ranging from 0.01 to 2.5 μg/L. The intra-assay and interassay coefficients of variation for this assay were 9.3% and 8.1%, respectively. The recovery of S100B added to CSF was 94%, and the lower limit of detection was 0.04 μg/L.

Determination of CSF Albumin and Total Protein Concentration

CSF albumin concentration was assayed by using rocket gel-electrophoresis with agarose gels containing monoclonal goat anti-human albumin antibody and human albumin as the calibrator. The total protein concentration was determined by using the benzthiazoline chloride method as previously described.21 ApoE Genotype Determination

APOE genotypes were determined by using DNA isolated from whole blood or CSF, amplified by using polymerase chain reaction, and digested for restriction fragment length polymorphism by using HhaI endonuclease.23

Evaluation of Clinical Outcome

Clinical outcome was determined by using the Glasgow Outcome Scale (GOS), assessed 3 months after SAH, and dichotomized into unfavorable recovery (GOS: 1 indicates death; 2, persistent vegetative state; and 3, severe disability) or favorable recovery (GOS: 4 indicates moderate disability; 5, good recovery).24

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism and InStat software (San Diego). Values of P < 0.05 were considered significant. These data were not normally distributed; therefore, group medians were compared by using the Mann-Whitney test. Correlation analysis was performed by using Spearman rank correlation, and the Fisher exact test was used to compare proportions.

TABLE 1. Characteristics of Patients With SAH

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SAH (n=19), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56, 54, 35–74</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male, female</td>
<td>6 (32), 13 (68)</td>
</tr>
<tr>
<td>APOE genotype</td>
<td></td>
</tr>
<tr>
<td>e33</td>
<td>11 (58)</td>
</tr>
<tr>
<td>e34</td>
<td>7 (37)</td>
</tr>
<tr>
<td>e32</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (5)</td>
</tr>
<tr>
<td>WFNS clinical grade</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (37)</td>
</tr>
<tr>
<td>II</td>
<td>2 (10)</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>6 (31)</td>
</tr>
<tr>
<td>V</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Fisher CT grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 (5)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>9 (47)</td>
</tr>
<tr>
<td>4</td>
<td>9 (47)</td>
</tr>
</tbody>
</table>

Age and GCS are given as mean, median, and range, respectively. GCS indicates Glasgow Coma Score; WFNS, World Federation of Neurological Surgeons.

within 24 hours of admission according to the grading scale of the World Federation of Neurological Surgeons for patients with SAH.16,17 The blood load on the admission CT scan was stratified according to the grading system of Fisher et al.18 These data are summarized in Table 1. Hemorrhage was due to rupture of an anterior circulation aneurysm in 17 patients and posterior circulation aneurysm in 2 patients.

The control subjects were selected from 229 consecutive CSF samples that were analyzed in the Department of Neuropathology, Institute of Neurological Sciences, Glasgow, UK, and had a nucleated cell count of <5/mm3. The group was composed of 28 patients (mean age 40 years, median 37 years, range 19 to 73 years) who had undergone an examination of the CSF by lumbar puncture for investigation of suspected neurological disease. Clinical criteria for selection included no history of acute brain injury or impaired conscious level, normal neurological examination, negative imaging studies, and no definitive diagnosis. Additional selection criteria included CSF albumin and total protein within 2 SD of the mean, absent oligoclonal bands, and no xanthochromia.

The present study had approval of the local hospital ethics committee, and written informed consent (assent obtained from next of kin of patients incapable of giving informed consent) was required for participation in the study.
Results

CSF Analysis
The median concentration of apoE in the CSF of patients with SAH was \( \approx 3 \) times lower than that of the control group (Table 2, Figure 1). The decrease in CSF apoE, observed within 3 days of SAH, was statistically significant \(( P<0.0001)\). The concentration of albumin and total protein in the CSF was significantly higher \(( P<0.0001)\) after SAH. Despite the increase in CSF albumin after SAH, the ratio of apoE to albumin was significantly decreased \(( P<0.0001)\) after SAH. In addition, relative to the increase in total protein observed in the CSF after SAH, apoE was significantly decreased \(( P<0.0001)\). The CSF concentration of the brain-specific protein S100B also increased significantly \(( P<0.0001)\) after SAH. These data are summarized in Table 2 and Figure 1.

CSF ApoE Correlation With Injury Severity and Clinical Outcome
There was a significant correlation between the concentration of apoE in the CSF after SAH and injury severity as assessed by the GCS (Spearman \( r=0.5, P<0.03, 95\% \) CI 0.04 to 0.78). CSF apoE was also correlated with clinical outcome, which was assessed 3 months after SAH by the GOS (Spearman \( r=0.53, P=0.018, 95\% \) CI 0.09 to 0.80) (see Figure 2A and 2B). The concentration of apoE in the CSF was significantly lower \(( P=0.03)\) in SAH patients with unfavorable outcome than in patients with favorable outcome (see Figure 2C). Compared with the proportion of patients with favorable outcome, the proportion of patients with unfavorable outcome and CSF apoE concentration below the lowest control value was significantly higher \(( P=0.02 \) by Fisher exact test). However, there was no significant difference between the proportion of patients with favorable versus unfavorable outcome and possession of the \( APOE \) e4 allele. There was no significant difference in the CSF apoE concentration for SAH patients possessing the \( APOE \) e4 allele \(( n=7)\) versus noncarriers of the \( APOE \) e4 allele. CSF S100B concentration was not correlated with apoE concentration, injury severity (by GCS), or outcome (by GOS) after SAH. There was no significant correlation between CSF apoE and S100B and Fisher CT grade.

Discussion
The main findings of the present study are that the concentration of apoE is substantially lower in the CSF of patients with SAH than in the CSF of noninjured control subjects and that after SAH there is weak correlation between CSF apoE concentration and both injury severity and clinical outcome. These findings are particularly striking because plasma is released into the subarachnoid space at the time of hemorrhage, and the concentration of apoE is \( \approx 10 \) times greater in plasma than in CSF.25 There are a number of possible mechanisms that might explain the decrease in CSF apoE after SAH. The possibility that the apoE signal is decreased because of assay interference from protein or lipid released after injury is not

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**TABLE 2. Concentration of ApoE, S100B, Albumin, and Total Protein in the CSF After SAH and Noninjured Controls**

<table>
<thead>
<tr>
<th>CSF Concentration</th>
<th>Control (n=28)</th>
<th>SAH (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE, mg/L</td>
<td>12.4 (12.1±4.7)</td>
<td>4.6 (3.9±3.8)</td>
</tr>
<tr>
<td>[0.34]</td>
<td>[0.13]</td>
<td></td>
</tr>
<tr>
<td>S100B, µg/L</td>
<td>0.39 (0.29±0.37)</td>
<td>19.9 (5.8±30.1)</td>
</tr>
<tr>
<td>[0.0185]</td>
<td>[1.5]</td>
<td></td>
</tr>
<tr>
<td>Albumin, mg/L</td>
<td>177 (171)±40</td>
<td>957 (359)±161</td>
</tr>
<tr>
<td>[2.7]</td>
<td>[14.5]</td>
<td></td>
</tr>
<tr>
<td>Total protein, g/L</td>
<td>0.32 (0.27±0.12)</td>
<td>1.97 (1.81±1.34)</td>
</tr>
</tbody>
</table>

Mean (median) CSF protein concentration±SD is given. Micromolar (µM) concentration is given in square brackets.

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**Figure 1.** Column scatterplot of CSF apoE, apoE/albumin, apoE/total protein ratio, and S100B in control subjects and in patients after SAH. Shown are concentrations in control and SAH CSF of apoE (A), S100B (B), apoE/albumin (C), and apoE/total protein (D). The horizontal bar represents the group median. From the Mann-Whitney test comparing the control and SAH groups, median concentrations are significantly \(( P<0.0001)\) different for all the proteins analyzed.
supported by the high recovery of apoE from control and SAH CSF. It is possible that the development of hydrocephalus after SAH accounts for some of the decrease in CSF apoE concentration that is due to dilution. However, it is unlikely that dilution alone accounts for the observed decrease because the increase in CSF volume necessary to produce this decrease would not be compatible with survival. Our understanding of lipoprotein flux within the CNS and between the CNS and periphery is far from complete, and the possibility that CSF apoE is decreased because of increased clearance to the plasma compartment warrants further investigation. The absence of correlation between CSF and plasma apoE concentration, coupled with the magnitude of the CSF-plasma apoE concentration gradient, suggests that the decrease in CSF apoE concentration observed after SAH is not attributable to the changes in plasma apoE concentration that might occur after SAH. The plasma concentration of other lipoproteins appears to remain stable in the acute phase after stroke.

In normal human brain, there is minimal transfer of apoE across the blood-brain-barrier, and CSF apoE is synthesized intrathecaally by astrocytes, microglia, and oligodendrocytes. In vitro apoE secretion by astrocytes is decreased in the presence of proinflammatory cytokines, the concentration of which increases in the CSF after SAH. Thus, it is possible that after SAH, release of apoE into the CSF is diminished as part of the inflammatory response to injury.

It is somewhat surprising that plasma apoE released into the subarachnoid space at the time of hemorrhage does not result in increased ventricular CSF apoE concentrations. Albumin from the plasma compartment, other proteins, and the predominantly CNS isoform of $S100$ are released into the ventricular CSF after SAH. The findings reported in the present study suggest that apoE is selectively retained within the subarachnoid space or neuropil. Alternatively, but not convincingly, CSF apoE clearance into the plasma compartment may be upregulated after SAH.

The concept that proteins may be eliminated from the interstitial fluid of the CNS at different rates is not novel and, in the case of insoluble peptides such as amyloid-$\beta$ protein (A$\beta$) or prion protein, may be relevant to the extracellular deposits identified in Alzheimer’s disease and Creutzfeldt-Jakob disease, respectively. Intriguingly, the concentration of these proteins appears to decrease in the CSF of patients with these disorders. There is evidence from human postmortem material that in brain injury due to trauma, apoE and A$\beta$ are codeposited in the cerebral cortex. Binding interactions between apoE and A$\beta$ resulting in the formation of insoluble aggregates have been reported in vitro. Consistent with this finding, in vivo studies with amyloid precursor protein and $APOE$ transgenic mice support a role for apoE as a promoter for the formation of fibrillar A$\beta$, amyloid plaque, and neurite dystrophy. Evidence from other studies supports the concept that there is an increase in brain apoE after injury. Studies of human postmortem tissue material identified increased immunoreactivity for apoE in the CNS parenchyma during the acute phase of several types of insult, including traumatic brain injury, herpes simplex encephalitis, demyelination, and hypoxic brain injury. Thus, after SAH, the formation of insoluble aggregates could be a mechanism explaining the findings in the present study. We speculate that apoE may be transported to the intracellular compartment as an apoE-A$\beta$ or cholesterol/phospholipid-
apoE complex (lipoprotein particle) by lipoprotein receptor-mediated endocytosis. In support of this concept, cells in contact with CSF and neurons of the cerebral cortex express lipoprotein receptors.43

Experimental models of acute brain injury have identified increased apoE immunoreactivity in the selectively vulnerable population of neurons, which appear to take up apoE in response to injury.9–11 It has been suggested that this reflects a major role for apoE in the clearance of cholesterol and lipid debris from areas of injury and recycling to neurons engaged in repair and regeneration.13,14,44 It has also been suggested that apoE may function as a modulator of oxidative stress.45 The correlation of low CSF apoE with high tissue concentration would further support the concept that apoE exhibits protective functions by limiting oxidative damage or efficient recycling of lipid.

In contrast to the decreased concentration of apoE in the CSF after SAH, albumin, total protein, and S100B increased markedly. It is not known whether the increased CSF S100B concentration observed after acute human brain injury reflects the passive release of S100B from damaged CNS cells or is the result of an active response of the astrocyte to injury.15 In the present small study, there was no correlation between CSF S100B and injury severity (by GCS) or clinical outcome (by GOS). There was no statistically significant association between the CT scan grade of injury severity and other data in the study. There was weak correlation between CSF apoE concentration and both injury severity and clinical outcome after SAH, supporting the concept that apoE is involved in the response of the brain to injury and or the recovery process itself.

There were a number of limitations to the present study. First, because of limited availability of CSF in brain-injured patients, the study population was small and selective, limiting the power to detect potential effects due to differences in APOE genotype, apoE concentration, S100B concentration, injury severity, and clinical outcome. Second, the extrapolation that CSF apoE is decreased after acute brain injury because of its selective retention in the brain may not be justified. In the absence of noninvasive methods for imaging these proteins in vivo or until invasive methods such as microdialysis have been adapted for this purpose, demonstrating increased parenchymal apoE concentrations will require analysis of tissue for which there is a clinical indication for surgical excision. Third, our analysis was restricted to CSF taken within 3 days of SAH, limiting kinetic analysis of the change in CSF apoE concentration. In addition, defining the normal concentration of a substance in normal CSF was problematic because of the restriction of CSF sampling to patients investigated for suspected neurological disease. The concentration of intrathecally synthesized proteins such as apoE is higher in CSF from the ventricles compared with CSF from the lumbar subarachnoid space; therefore, the magnitude of the decrease in apoE that we report is likely to represent an underestimation.

Despite the limitations of the present study, these preliminary findings provide new evidence that apoE concentration in the CSF falls substantially in patients surviving SAH. This observation supports the hypothesis that apoE plays an important role in the response of the CNS to injury, possibly involving scavenging of cholesterol and lipids from injured tissue for potential recycling or modulation of oxidative stress.

In conclusion, decreased concentration of apoE in the CSF after SAH and the correlation of injury severity and clinical outcome provide new in vivo evidence that apoE is involved in the response of the human CNS to injury. The role that apoE plays in this context is uncertain, but further understanding may result in novel treatment strategies for the treatment of acute brain injury.

Acknowledgments

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References


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