Fas System Activation in Perihematomal Areas After Spontaneous Intracerebral Hemorrhage

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Background and Purpose—Apoptosis has been implicated as the prominent form of cell death in the brain perihematomal region in animal models and in autopsy or postsurgical human studies. Both the Fas system and caspase activation play a central role in apoptotic pathways. The aims of this study were to investigate soluble Fas (s-Fas) plasma levels after acute intracerebral hemorrhage (ICH), to determine its influence on clinical and radiologic features, and to assess Fas receptor and Fas ligand (Fas-L) protein expression in human ICH brain tissue.

Methods—s-Fas plasma levels were determined on admission in 78 consecutive ICH patients and serially in a subgroup of 21 of them, at the time of neurologic assessment, by means of ELISA. ICH and perihematomal edema volumes were determined at baseline and on follow-up computed tomography scans, and ICH and perihematomal edema growth was calculated. The presence of Fas receptor and Fas-L was assessed in different brain tissue samples by immunoblotting from 6 deceased ICH patients and from 2 control subjects.

Results—Mortality reached 20.5% of patients at the third month, and 48% of survivors had an unfavorable outcome (modified Rankin Scale score ≥3). The baseline s-Fas level in ICH patients was significantly lower than in healthy controls [160 (160–245) vs 269 (230–332) pg/mL, P<0.001], returning to normal values by 24 hours (P<0.05 for all determinations). Regarding radiologic features, the baseline s-Fas value was found to be inversely correlated to perihematomal edema growth at follow-up (r = −0.33, P=0.041). Finally, Fas-L content was highest in the perihematomal area compared with contralateral and remote ipsilateral areas in ICH patient and control samples.

Conclusions—A decreased plasma s-Fas level together with an increased Fas-L amount in perihematomal brain tissue suggest Fas-mediated apoptosis involvement in this disease. (Stroke. 2008;39:1730-1734.)

Key Words: apoptosis ▪ intracerebral hemorrhage ▪ edema ▪ Fas receptor ▪ Fas ligand ▪ soluble Fas ▪ stroke

Intracerebral hemorrhage (ICH) induces several histopathologic changes in its proximity, which in addition to a mass effect, are responsible for brain damage.1 Among them, cellular death has been shown to be caused by either necrosis or apoptosis-related mechanisms. Necrosis usually occurs as a passive process involving a group of cells and produces a significant inflammatory response. In contrast, apoptosis affects actively single cells, with the formation of apoptotic bodies and their phagocytosis by adjacent normal cells. The characterization of cell death associated with ICH has been previously defined in several experimental models,2–4 which showed that apoptosis occurs not only in the matrix of the hematoma but also in the tissue surrounding its periphery. Qureshi et al5 also identified the presence of apoptotic cells in the perihematomal region of surgically evacuated human hematomas.

Apoptosis requires the activation of a cell death gene program and implies the activation of extracellular signals that can regulate this process, which ends with caspase-3 activation directly related to DNA damage and cell death.6 The Fas (also called Apo-1 or CD95) system is an apoptosis-signaling receptor located on the cell surface that belongs to the tumor necrosis factor receptor family.7 The interaction between Fas and its ligand (Fas-L) leads to apoptosis. However, several Fas variant molecules have been identified, which are generated by alternative splicing and are expressed as soluble molecules, some of them capable of inhibiting the induced apoptosis.8 Fas-mediated apoptosis has been described in other central nervous system diseases, such as traumatic brain injury, multiple sclerosis, and ischemic stroke,9–13 although its role in ICH has not been yet defined.

Our aim was to investigate the presence of soluble Fas (s-Fas) in plasma samples after acute ICH and to determine its relation to clinical and radiologic features. In addition, the presence of Fas receptor and Fas-L were also investigated in human ICH brain tissue.
Subjects and Methods

Study Population
Our target population consisted of consecutive patients with spontaneous supratentorial ICH evaluated in the Emergency Department within the first 24 hours after onset. A detailed history of vascular risk factors, drug abuse, alcoholism, liver disease, and concomitant medication was obtained from each patient. All patients with ICH related to vascular malformation, impaired coagulation or oral anticoagulant intake, traumatic brain injury, hemorrhagic infarction, and tumoral bleeding and those patients who underwent a surgical procedure were excluded from this study. Finally, a total of 78 ICH patients were studied after informed consent was obtained from them or their relatives. The ethics committee of the hospital approved all aspects of the study protocol.

Clinical Assessment
The Glasgow Coma Scale and National Institutes of Health Stroke Scale (NIHSS) scores were recorded to assess the level of consciousness and neurologic status on admission (<24 hours) and at follow-up (24 hours, 48 hours, 7 days, and 3 months) visits.

Early neurologic worsening (defined as an increase in NIHSS score by ≥4 points within the first 48 hours) was recorded. Mortality, or a modified Rankin Scale score ≥3 among survivors, was considered an unfavorable outcome parameter at the third month.

Arterial blood pressure and temperature values were obtained immediately before the computed tomography (CT) scan and at follow-up visits. Blood glucose values and leukocyte counts were also recorded at the baseline visit.

CT Scan Protocol
Two cranial CT scans were carried out, on admission (<24 hours) and at follow-up (median, 3 days). All cranial CT scans were performed according to the Neuroradiology Department protocol, with an image matrix of 340×340 and 2.5-mm slice width for the posterior fossa and 10 mm for the next slices. Investigators who read the CT scans were blinded to clinical and laboratory information. ICH volume was measured on baseline and follow-up cranial CT scans, according to the formula A×B×C×0.5, where A and B represent the largest perpendicular diameters through the hyperdense area on the CT scan and C represents the thickness of the ICH (ie, the number of 10-mm slices containing the hemorrage). ICH location was categorized as deep, when it was limited to the basal ganglia and/or the thalamus, or lobar, when it affected predominantly the subcortical white matter of the cerebral lobes. A hematoma growing by >33% of its initial volume was considered hematoma enlargement, according to previous published criteria.

Perihematomal edema (PE) volume was also measured on baseline and follow-up CT scans by subtracting the hyperdense volume (ICH area) from the total lesion area, according to the formula mentioned earlier. PE enlargement on the follow-up CT scan was measured as described for PE volume, but the clot retraction volume, if any, was subtracted. We considered edema enlargement when there was an increase of at least 33% compared with the baseline CT scan. Finally, the presence of intraventricular or subarachnoid extensions of the hematoma were also recorded at the time of the baseline and follow-up CT scans.

Immunooassay Methods
Venous blood samples were drawn from each patient on admission (n=78) and at follow-up visits (n=21). EDTA tubes were used to collect the blood, and plasma was immediately separated by centrifugation at 3000 rpm for 15 minutes and stored at –80°C until analysis. The s-Fas level was determined with use of a commercially available ELISA according to the manufacturer’s instructions (Bender Medsystems, Vienna, Austria). Our laboratory reference range for healthy controls was 269 (230–332) pg/mL.

Brain Tissue Samples
We studied a total of 18 samples from 6 patients with fatal ICH, as well as those from 2 control subjects who died of other noninflammatory, nonneurologic diseases. Sampling was done as previously described. In brief, on autopsy, the blood mass was removed, and 1 cm³ of adjacent parenchyma was obtained as a perihematomal area, together with contralateral tissue. We also obtained an additional area remote from the ICH in the same hemisphere. Sampling was done within the first 6 hours after death to avoid tissue degradation. All brain parenchyma was snap-frozen in LN₂ and stored at –80°C.

Immunoblotting
Supernatant homogenates were obtained for Fas receptor and Fas-L Western blot analysis, and protein content was previously determined by bicinchoninic acid assay. In brief, 30 μg total protein was loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane. Nonspecific bindings were blocked, and membranes were incubated separately overnight with rabbit anti-human Fas receptor (Alexis) and rat anti-human Fas-L at 1:1000 (Alexis). Secondary biotinated antibody (Chemicon, Temecula, CA) was used, and membranes were incubated for 1 hour at room temperature followed by a final streptavidin–horseradish peroxidase incubation (1:1000) for 1 hour. Before and after membrane incubations, 3 washes (10 minutes each) in Tris phosphate-buffered saline were done. The substrate reaction was developed with the chemiluminescent reagent ECL PLUS (Amersham) and visualized with a luminescent image analyzer (Gel Logic 440 Imaging System, Kodak).

Immunohistochemistry
To localize the Fas receptor, frozen perihematomal tissues were carefully embedded in OCT compound in a cryostat holder, and 10-μm sections were placed on precoated polylysine-coated slides and stored at –80°C. Sections were air-dried, fixed in cold acetone for 15 minutes, and rehydrated with Tris-buffered saline for 5 minutes. Bovine serum albumin (1%) was applied, and the sections were incubated at room temperature for 30 minutes. Incubation with the primary antibody, mouse anti-human Fas 1:20 (Alexis), was carried out overnight at 4°C. Peroxidase activity was blocked with 3% H₂O₂ in Tris-buffered saline for 30 minutes. The secondary antibody (1:500, peroxidase-linked sheep anti-mouse, Amersham) was applied for 1 hour at room temperature. Immunoreactive sites were developed with diamobenzidine solution applied for 15 minutes, and sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in DPX. As negative controls, only the secondary antibody was applied to some sections to detect nonspecific immunoreactivity.

Statistical Analysis
Statistical analysis was done with the SPSS 12.0 statistical package. Statistical significance for intergroup differences was assessed by Fisher’s exact test for categorical variables and the Mann–Whitney U test for continuous variables. To study correlations between continuous variables, Spearman and Pearson correlation coefficients were used as required. A repeated-measures ANOVA and a logistic-regression analysis was performed to adjust factors related to edema enlargement by using a forward stepwise method with a probability value <0.05 was considered statistically significant.

Results
Clinical and Neuroimaging Findings
Demographic data, risk factor profile, and clinical variables are shown in Table 1, and radiologic data from baseline and follow-up CT scans are provided in Table 2. Mean baseline
ICH volume and mean follow-up ICH volume from the patients with fully available data were 16.4 and 18 cm³, respectively.

At follow-up, 13 patients showed neurologic deterioration, and 16 had died as a consequence of ICH by the third month. Among all survivors, 48% had an unfavourable outcome (modified Rankin Scale score ≥3). Neurologic worsening was associated with a lower Glasgow Coma Scale score and a higher baseline leukocyte count, whereas mortality was found to be related to baseline ICH volume, intraventricular extension, and leukocyte count.

Approximately 30% of patients with an available follow-up CT scan demonstrated hematoma enlargement. Edema enlargement was observed in up to 51% of patients during the first 3 days. Hematoma or edema enlargement was not recorded in 12 patients who died within the first few days and in 23 patients who were scanned beyond the third day.

Table 1. Demographic Data, Risk Factor Profiles, and Clinical Variables Among the Study Patients

<table>
<thead>
<tr>
<th></th>
<th>ICH (N=78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male</td>
<td>53 (68%)</td>
</tr>
<tr>
<td>Age, y</td>
<td>75 (63.5–80)</td>
</tr>
<tr>
<td>Previous high blood pressure</td>
<td>50 (64%)</td>
</tr>
<tr>
<td>Smokers</td>
<td>19 (24%)</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>16 (20%)</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>17 (22%)</td>
</tr>
<tr>
<td>Ischemic</td>
<td>11 (14%)</td>
</tr>
<tr>
<td>ICH</td>
<td>6 (7%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>22 (28%)</td>
</tr>
<tr>
<td>Liver disease</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>Current or active antiplatelet therapy</td>
<td>26 (33%)</td>
</tr>
<tr>
<td>Baseline Glasgow Coma Scale score</td>
<td>15 (14–15)</td>
</tr>
<tr>
<td>Baseline NIHSS score</td>
<td>12 (6–16)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>176 ± 29</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>95 ± 21</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>36.4 ± 0.4</td>
</tr>
</tbody>
</table>

Data are expressed in n (%), median (interquartile range), or mean ± SD, as appropriate.

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Table 2. Data From Baseline and Follow-Up CT Scans

<table>
<thead>
<tr>
<th></th>
<th>Total Series (n=78)</th>
<th>Patients With Imaging Follow-Up (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to baseline CT, h</td>
<td>5.2 (2–10)</td>
<td>5.4 (2.1–10.1)</td>
</tr>
<tr>
<td>Location (lobar)</td>
<td>24 (31%)</td>
<td>12 (27.9%)</td>
</tr>
<tr>
<td>Baseline ICH volume, cm³</td>
<td>18 (5–39)</td>
<td>16.4 (6.7–37.5)</td>
</tr>
<tr>
<td>Baseline PE volume, cm³</td>
<td>5 (0.4–16)</td>
<td>5.3 (0.2–14.6)</td>
</tr>
<tr>
<td>Presence of intraventricular extension</td>
<td>20 (25%)</td>
<td>9 (21%)</td>
</tr>
<tr>
<td>Presence of subarachnoid extension</td>
<td>5 (6%)</td>
<td>2 (4.6%)</td>
</tr>
<tr>
<td>Follow-up ICH volume, cm³</td>
<td>...</td>
<td>18 (7.5–47)</td>
</tr>
<tr>
<td>Follow-up PE volume, cm³</td>
<td>...</td>
<td>13 (6–37)</td>
</tr>
</tbody>
</table>

Time to baseline CT is the time from stroke onset to baseline CT scan in hours. All volumes are expressed as cm³. Data are expressed in n (%) and median (interquartile range), as appropriate.

s-Fas Temporal Profile

Baseline s-Fas level among ICH patients was significantly lower than among healthy controls [160 (160–245) vs 269 (230–332) pg/mL; P<0.001]. Moreover, the temporal profile of s-Fas (Figure 1) showed that the baseline s-Fas level was also lower than the remaining follow-up determinations (P<0.05 for all groups). In fact, s-Fas had returned to normal at the 24-hour determinations.

Figure 1. Box-and-whisker plot of s-Fas levels in control subjects and ICH patients. Baseline s-Fas concentrations were significantly lower in ICH patients than in controls and after 24 hours, 48 hours, 7 days, and 3 months. *P<0.05.

Figure 2. Scatterplot showing the inverse relation between baseline s-Fas levels and edema enlargement during follow-up.
Clinical and Radiologic Features Related to s-Fas Level
The baseline s-Fas level was not related to any clinical or biologic variable on admission or follow-up. Regarding radiologic features, the s-Fas level was not related to initial ICH and PE volumes, nor with the presence of intraventricular or subarachnoidal extension. However, during follow-up, as shown in Figure 2, a negative correlation emerged between baseline s-Fas level and PE enlargement in the following days \((r = -0.33, P = 0.041)\). Patients with edema enlargement had higher blood glucose values and higher baseline NIHSS scores and presented more often with hypertension as a vascular risk factor than those patients without edema enlargement. From all of these variables, only baseline s-Fas (odds ratio = 0.125; 95% CI, 95% 0.022 to 0.715; \(P = 0.02\)) remained a significant predictor of edema enlargement.

Fas Receptor and Fas-L Immunoblotting
Table 3 summarizes the clinical characteristics of patients included in the autopsy study. Western blot analyses, as shown in Figure 3, detected a 37-kDa band corresponding to Fas receptor protein. Overall, Fas receptor content was similar in all study samples (perihematomal areas, ipsilateral areas remote from the ICH, and contralateral areas), without significant differences among them \((P = 0.54)\). Fas receptor expression was absent in all study samples from control subjects (data not shown). Regarding Fas-L (31-kDa band), we found a significantly higher expression from the perihematomal areas compared with the contralateral and remote ipsilateral areas \((P < 0.001)\).

Fas Receptor Immunoreactivity
Finally, Fas receptor immunoreactivity was observed in the soma of cells determined morphologically as neurons by their

Table 3. Subjects Studied by Immunoblotting and Immunohistochemistry

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, y/Sex</th>
<th>Time, h, Stroke Exitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74/Male</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>84/Male</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>88/Male</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>74/Male</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>74/Female</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>82/Male</td>
<td>24</td>
</tr>
<tr>
<td>Control 1</td>
<td>64/Female</td>
<td>...</td>
</tr>
<tr>
<td>Control 2</td>
<td>82/Male</td>
<td>...</td>
</tr>
</tbody>
</table>
large size, triangular nucleus, and/or cytoplasm (supplemental Figure I, available online at http://stroke.ahajournals.org). Moreover, some of these stained cells showed morphological features corresponding to apoptotic neurons, such as shrunken cytoplasm and pyknotic nuclei.

Discussion

Although the potential role for apoptosis in cell death after ICH has been previously suggested, the molecular mechanisms underlying the induction of apoptotic cell death remain to be elucidated. Our results suggest the existence of Fas-mediated apoptosis involvement after ICH in perihematomal areas. In our study, s-Fas, which is thought to inhibit Fas interaction with its ligand, was decreased within the first 24 hours after ICH. This fact is in agreement with other studies involving cerebral ischemia, like that reported by Tarkowski et al. In their study, s-Fas level was decreased in cerebrospinal fluid from ischemic stroke patients and was inversely correlated with brain infarct volume and neurologic deficit 3 weeks and 3 months after the event. In our case, s-Fas was not correlated with the clinical status of our patients, but an association emerged between s-Fas and PE enlargement in the following days.

Several hypotheses have been suggested for edema formation. Most evidence from recent years has pointed to the existence of a hyperperfused, rather than an ischemic, area surrounding the clot. In this context, apoptosis could be an important mechanism of cell death in the perihematomal area, as it occurs in the peri-infarct area after ischemic stroke. In this context, whether the s-Fas initial decrease that we found reflects decreased production or rapid consumption remains to be determined. However, in our study, s-Fas levels returned to normal after the first 24 hours, so it seems to be a transient phenomenon. Also in our study, Fas receptor and particularly Fas-L were overexpressed in the areas immediately surrounding the clot, although other remote areas were also affected. Therefore, it is possible that activation of the Fas/Fas-L system contributes to brain damage by neuronal programmed death.

Recently, inhibition of this system has shown benefit in other central nervous system diseases. Subarachnoid administration of s-Fas receptor resulted in increased neuronal and oligodendrocyte survival and improved neurologic outcome in an in vivo model of spinal cord injury. Inhibition of Fas-L has also been shown to reduce secondary brain damage in experimental models of brain ischemia. A limitation of our study should be noted, as stroke severity and high mortality rates reduced the number of fully available neuroimaging data in our study subjects.

In conclusion, our findings extend current knowledge about the mechanisms of cell death underlying ICH. Moreover, our results might have therapeutic applications, such as the potential use of s-Fas in ICH treatment.

Disclosures

None.

References

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