Plasma Cellular-Fibronectin Concentration Predicts Hemorrhagic Transformation After Thrombolytic Therapy in Acute Ischemic Stroke

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Background and Purpose—Elevated plasma levels of cellular fibronectin (c-Fn) reflect vascular damage, so c-Fn might be a marker of secondary bleeding risk in cerebral ischemia. We investigated whether high plasma levels of c-Fn were associated with hemorrhagic transformation (HT) after treatment with tissue plasminogen activator (tPA) in patients with acute stroke.

Methods—Eighty-seven patients (mean age: 67±12) received tPA after the ECASS II criteria (mean time to infusion: 160±46 minutes; median NIHSS: 12). HT and hypodensity volume were studied on computed tomography (CT) performed 24 to 36 hours after treatment. HT was classified according to the ECASS II definitions. c-Fn and matrix metalloproteinase 9 (MMP-9) levels were determined by ELISA in blood samples obtained before treatment and in 30 healthy subjects.

Results—HT was found in 26 patients (30%); 15 patients had hemorrhagic infarction type 1 (HI-1), 7 had HI-2, and 4 had parenchymal hemorrhage (PH). Median c-Fn concentrations were 1.3, 1.7, 4.2, 5.4, and 7.3 μg/mL in controls, non-HT, HI-1, HI-2, and PH groups, respectively (P<0.001); median MMP-9 values were 54, 87, 154, 176, and 225 ng/mL (P<0.001). Logistic regression analysis showed that only c-Fn plasma levels remained independently associated with HT after adjusting for potential confounders (OR, 2.1; 95% CI, 1.3 to 3.4; P=0.002). Similar results were obtained in the 71 patients treated within 3 hours.

Conclusions—High plasma c-Fn levels are significantly associated with subsequent HT in stroke patients treated with tPA, so plasma c-Fn determinations might be useful in clinical practice to improve the risk/benefit ratio of thrombolytic treatment. (Stroke. 2004;35:1671-1676.)

Key Words: stroke ■ stroke, acute ■ hemorrhage ■ thrombolytic therapy

Thrombolytic therapy has been proven to be effective for the treatment of acute ischemic stroke, but the increased risk of hemorrhagic transformation (HT) associated with tissue plasminogen activator (tPA) administration is still of great clinical concern.1,2 HT after cerebral ischemia seems to be related to the disruption of the vascular endothelium.3 In patients who receive tPA treatment, endothelial injury may be the result of free radical generation secondary to thrombolytic-induced reperfusion,4 as well as of the upregulation of matrix metalloproteinases (MMPs),5 a group of enzymes that are able to degrade the basal membrane components. The association between high levels of MMP-9 and the risk of HT in patients with acute ischemic stroke who have6 and have not7 received tPA have been previously reported. However, despite the available data, the underlying molecular mechanisms related to HT after thrombolytic treatment have yet to be fully elucidated.

Fibronectins are adhesive dimeric glycoproteins that promote cell–cell and cell–matrix interactions.8 Plasma fibronectin (p-Fn) is primarily produced by hepatocytes,8 but plasma also contains small quantities of cellular fibronectin (c-Fn), which is mainly synthesized by endothelial cells.9 Because c-Fn is largely confined to the vascular endothelium, high plasma levels of this molecule might be indicative of endothelial damage. In fact, plasma c-Fn levels have been reported to be increased in patients with vascular injury secondary to vasculitis, sepsis, acute major trauma, diabetes, and patients with ischemic stroke.10,11

Because HT after cerebral ischemia seems to be the result of the continuous disappearance of basal membrane components,3 in the present study we sought to investigate whether high levels of plasma c-Fn were associated with HT in patients who received thrombolytic treatment with tPA.

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Subjects and Methods
We prospectively studied 87 patients (59% men; mean age 67±12 years) admitted consecutively into 2 university hospitals between October 1999 and July 2001 who received intravenous tPA following the European Cooperative Acute Stroke Study (ECASS) II criteria.12 Thrombolytic therapy was administered within 6 hours from the beginning of the symptoms at a dose of 0.9 mg/kg body weight, with an upper dose limit of 90 mg per patient. Ten percent of the total dose was given as a bolus over 1 to 2 minutes, followed by a 60-minute infusion of the remaining dose. The mean time to the infusion of the drug was 160±46 minutes. Seventy-one patients received the treatment within 3 hours from onset of symptoms, whereas 16 patients received tPA between 3 and 6 hours within onset of symptoms. Thirty healthy control subjects matched by age and sex (male: 57%; mean age: 63±9 years) and without history of neurological disorders or vascular risk factors were also included in the study. To determine the effect of stroke on the levels of the molecules, plasma c-Fn and MMP-9 concentrations were also determined in 100 patients with acute ischemic stroke who did not receive tPA treatment and in whom HT did not develop (male: 59%; mean age: 67±6 years; mean time to inclusion: 7.2±3.9 hours).

Neither the patients nor the controls had inflammatory, hematological, or infectious diseases, cancer, or severe renal or liver failure. The ethics committee approved the protocol in each center, and informed consent was obtained from patients or their relatives.

Medical history recording potential stroke risk factors, clinical examination, blood and coagulation tests, 12-lead electrocardiogram, chest radiography, and noncontrast cranial computed tomography (CT) scan were performed at admission. Stroke subtype was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.13 Stroke severity was assessed by a certified neuroradiologist using the National Institutes of Health Stroke Scale (NIHSS) at admission and at 24 to 36 hours. Neurological deterioration was defined as death or an increase of ≥4 points in the NIHSS score between the 2 examinations.

Early CT signs of infarction were evaluated in the first radiological examination. The volume of hypodensity and the presence of HT were evaluated on a second cranial CT, which was performed 24 to 36 hours after treatment. Hypodensity volume was determined using the formula 0.5ab²/2c, where a and b are the largest perpendicular diameters as displayed on CT and c is the slice thickness.

The HT type was classified according to the ECASS II criteria.12 Hemorrhagic infarction type 1 (HI-1) was defined as small petechiae along the margins of the infarct, and HI type 2 (HI-2) was defined as more confluent petechiae within the infarct area but without a space-occupying effect. Parenchymal hemorrhage type 1 (PH-1) was defined as blood clots in <30% of the infarcted area with some slight space-occupying effect, and PH type 2 (PH-2) as blood clots in >30% of the infarcted area with substantial space-occupying effect.

All CT examinations were performed by 1 investigator in each center blinded to the clinical and analytical data. We considered symptomatic HT as being associated with neurological deterioration.

Laboratory Tests
Blood samples were taken from all patients at admission before tPA administration. Samples were collected in glass test tubes containing EDTA. Suspension of plasma was centrifuged at 3000g for 5 minutes and immediately frozen and stored at −80°C. Plasma MMP-9 and c-Fn levels were measured with commercially available quantitative sandwich enzyme-linked immunosorbent assay kits obtained from Biotrak, Amersham Pharmacia UK, and Adera Biomedical, respectively. Determinations were performed in an independent laboratory blinded to clinical and radiological data. The intra-assay and interassay coefficients of variation were <5% for MMP-9 and c-Fn determinations.

Statistical Analysis
Proportions between groups were compared using the χ² test. Continuous variables are expressed as mean±SD and were compared using the Student t test. Given that MMP-9 and c-Fn concentrations are not normally distributed, their levels were expressed as median (quartiles), and comparisons were made using the Mann–Whitney test or Kruskal–Wallis test as appropriate.

The association between c-Fn levels and baseline continuous variables was assessed by calculating the Spearman correlation coefficient. We used cutoff values, as described by Robert et al.,14 to estimate the sensitivity, specificity, and predictive values of a specific concentration of plasma MMP-9 and c-Fn for HT. The importance of MMP-9 and c-Fn in the development of HT after tPA administration was determined by logistic regression analysis after adjusting for those variables evaluated at admission that were related to HT in the univariate analysis. Because plasma levels of c-Fn have been reported to increase with age10 and in patients with diabetes,13 these 2 variables were forced into the analysis. To test whether the odds of HT for c-Fn was modified by the volume of hypodensity, a second analysis was performed including this factor into the model. Plasma MMP-9 and c-Fn were included as continuous variables because the cutoff values meant that there was a linearity of the odds ratios.

Results
Twenty-six (30%) of the 87 patients included in the study had HT. Fifteen patients (17.2%) had HI-1, 7 (8%) had HI-2, 2 (2.3%) had PH-1, and 2 (2.3%) had PH-2. Table 1 shows the main characteristics of patients with and without HT. The severity of neurological deficit at admission evaluated by the NIHSS score was significantly higher in patients with HT, who also displayed significantly greater volumes of hypodensity on the second cranial CT. Both clinical groups presented with similar systolic and diastolic blood pressures and glucose levels before tPA administration. No statistically significant differences were found in the presence of early signs of cerebral infarction at admission or in the stroke mechanism in patients with and without HT.

Neurological deterioration was observed in 15 patients (17.2%). In 8 patients, the neurological worsening was associated with HT: 2 patients had HI-1, 3 displayed HI-2, and 3 had PH.

Plasma c-Fn concentrations before tPA administration were significantly higher in patients with HT (4.8 [3.4, 5.9] μg/mL) than in those without HT (1.7 [1.4, 2.5] μg/mL) and both the healthy subjects (1.3 [0.9, 1.6] μg/mL) and the patients not treated with tPA (1.4 [1.1, 1.8] μg/mL) (all P<0.001). Moreover, we found that the greater the severity of the bleeding the higher the levels of c-Fn (Figure 1A). A similar effect was found in those patients who were treated within 3 hours of onset of symptoms (n=71) (Figure 1B). The levels of c-Fn were not statistically different in patients with symptomatic and asymptomatic HT, although there was a clear trend for the levels to be higher in patients with symptomatic HT (5.8 [4.0, 6.9] μg/mL versus 4.5 [2.7, 5.4] μg/mL; P=0.054).

Plasma MMP-9 concentrations before tPA administration were also significantly higher in those patients with HT (170.3 [101.4, 196.2] ng/mL) than in those without HT (87.2 [54.8, 115.1] ng/mL) and in both the healthy subjects (53.7 [39.5, 79.4] ng/mL) and the patients not treated with tPA (62 [40, 93.8] ng/mL) (all P<0.001). As observed with c-Fn levels, the greater the severity of the bleeding, the higher the levels of MMP-9, both in patients treated within 6 (Figure 2A) and 3 hours (Figure 2B). No differences were found in MMP-9 levels between symptomatic and asymptomatic HT.
Plasma c-Fn concentrations were significantly higher in patients with early signs of ischemia \((n=37)\) on cranial CT \((2.8 [1.7, 5.0] \mu g/mL)\) than in those without \((1.9 [1.4, 2.9] \mu g/mL)\) \((P=0.012)\). Plasma c-Fn correlated positively with MMP-9 levels \((r=0.671, P<0.001)\) and the hypodensity volume \((r=0.364, P<0.001)\). No correlation was found between plasma c-Fn levels and other variables related to HT such as serum glucose concentrations, blood pressure levels, or the severity of neurological deficit at admission.

As shown in Table 2, only plasma c-Fn levels remained independently associated with HT after adjustment for age, history of diabetes, baseline NIHSS score, and plasma MMP-9 levels. The odds of c-Fn levels for HT did not substantially change after the inclusion of the volume of hypodensity into the analysis \((OR, 2.1; 95\% CI, 1.3 to 3.4; P=0.002)\). Plasma c-Fn level was also the only factor independently associated with HT after adjustment for potential confounders when the analysis was limited to the 71 patients treated within 3 hours \((OR, 1.9; 95\% CI, 1.2 to 3.3; P=0.006)\). No interaction was found between MMP-9 and c-Fn.

Because it has been reported that HI-2 and PH occur more often in patients who receive tPA treatment, and also based on the observation of our own results in Figure 1 that clearly demonstrate higher levels of c-Fn in patients with HI-2 and PH, we calculated the c-Fn and MMP-9 cutoff values with the highest sensitivity and specificity for these types of HT. This post-hoc explanatory analysis showed that plasma MMP-9 concentrations \(\geq 140 \mu g/mL\) predicted the development of HI-2 and PH with a sensitivity of 81%, specificity of 88%, positive predictive value of 41%, and negative predictive value of 98%. More interestingly, the sensitivity, specificity, and positive and negative predictive values of plasma c-Fn \(\geq 3.6 \mu g/mL\) for the prediction of HI-2 and PH were 100%, 96%, 44%, and 100%, respectively.

### Discussion

Because thrombolytic therapy is the only treatment for ischemic stroke proven to be effective, the investigation of the underlying mechanisms responsible for HT, the most feared complication associated with this therapy, as well as the identification of factors that can improve the benefit/risk ratio of tPA administration is of critical importance. This study demonstrates that plasma c-Fn levels are significantly higher in patients in whom HT develops after tPA administration and suggests that c-Fn levels \(\geq 3.6 \mu g/mL\) can predict the development of HI-2 and PH after tPA administration with a sensitivity and negative predictive value of 100%. Therefore, c-Fn might be a useful marker of those patients.
who are at greatest risk for HT after the administration of thrombolytic treatment.

The loss of microvascular integrity secondary to the continuous disappearance of the antigens of the endothelial components has been reported as being responsible for HT after ischemic injury. Among these antigens, c-Fn is especially important because it mediates the interaction between the endothelium and blood cells as well as other blood components. Moreover, Fn plays an important role in blood clot formation by mediating the adhesion of platelets to fibrin, so the disappearance of the c-Fn of the vascular endothelium secondary to ischemia might damage this clotting mechanism, facilitating HT development. Although high c-Fn levels have been previously reported in patients with ischemic stroke, no previous data are available on the association between c-Fn levels and HT in patients with acute ischemic stroke.

The increase of vascular permeability and subsequent extravasation of serum components leading to HT after tPA administration may be the result of several mechanisms including the activation of MMPs, which is secondary to ischemia, and the administration of tPA. The present study confirms the significant association between MMP-9 levels and HT previously reported both in patients who received tPA and in a nonselected series of ischemic stroke patients. However, the fact that c-Fn is almost exclusively located at the endothelium suggests that this

### TABLE 2. Adjusted Odds Ratios of Hemorrhagic Transformation in Patients Treated Within 6 Hours From Symptoms Onset

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.0 (0.9–1.1)</td>
<td>0.780</td>
</tr>
<tr>
<td>History of diabetes</td>
<td>0.8 (0.2–3.6)</td>
<td>0.740</td>
</tr>
<tr>
<td>NIHSS score at admission</td>
<td>1.1 (0.9–1.3)</td>
<td>0.071</td>
</tr>
<tr>
<td>MMP-9 levels (by 10-unit increase, ng/mL)</td>
<td>1.1 (0.9–1.3)</td>
<td>0.086</td>
</tr>
<tr>
<td>c-Fn levels (μg/mL)</td>
<td>2.1 (1.3–3.3)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Age and history of diabetes were forced into the analysis. CI indicates confidence interval.

**Figure 1.** Box plots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (lines drawn from the end of the box) of baseline plasma c-Fn levels at admission in patients who received tPA within 6 hours (A) and 3 hours (B). *P<0.001 (Kruskal–Wallis test). HT indicates hemorrhagic transformation; HI-1, hemorrhagic infarction type 1; HI-2, hemorrhagic infarction type 2; PH, parenchymal hematoma.

**Figure 2.** Box plots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (lines drawn from the end of the box) of baseline plasma MMP-9 levels at admission in patients who received tPA within 6 hours (A) and 3 hours (B). *P<0.001 (Kruskal–Wallis test).
molecule could be a more specific marker of a high risk for HT. This hypothesis is supported by our finding that c-Fn levels, but not MMP-9 levels, remained independently associated with HT in the logistic regression analysis. Moreover, the predictive capacity of plasma c-Fn levels for the development of HI-2 and PH was higher than the predictive capacity of MMP-9 levels. However, although the difference did not reach statistical significance, probably because of the small sample size, there was a clear trend for the levels of c-Fn to be higher in patients with symptomatic HT, whereas MMP levels were similar in symptomatic and asymptomatic bleedings. Because neurological deterioration usually occurs in patients with more severe HT, c-Fn levels probably reflect not only endothelial damage but also the degree of endothelial damage. In agreement with this hypothesis, we have observed a positive correlation between c-Fn levels and hypodensity volume at 24 to 36 hours of evolution of the ischemia, which probably reflects the relationship between endothelial and brain injuries. This positive correlation could lead us to argue that c-Fn levels are just an epiphenomenon of the extent of brain damage. However, we find that it is the plasma c-Fn concentrations rather than infarct volume that independently predicted HT.

The origin of the elevated plasma levels of c-Fn after brain ischemic injury remains to be elucidated. The basal lamina disruption and the subsequent release of this molecule into the plasma, as well as accelerated Fn synthesis by endothelial cells and other cells such as polymorphonuclear leukocytes, arriving at the ischemic tissue as part of the ischemic inflammatory cascade, could be among the participating mechanisms. Interleukins and transforming growth factor β, whose expression is increased as a result of ischemia, have been shown to stimulate Fn synthesis. Increased c-Fn synthesis could be an attempt to decrease endothelial destruction by MMPs, which might explain the positive correlation between c-Fn and MMP-9 we observed. Experimental studies have demonstrated that endothelial cell injury leads to an increase in Fn production to provide re-endothelialization, and that the administration of synthetic Fn peptide V reduces the final infarct volume of cerebral ischemia when administered within 3 hours after reperfusion.

The present study has some limitations. First, although we have demonstrated the accuracy of plasma c-Fn levels ≥3.6 µg/mL for the prediction of HI-2 and PH, these data were obtained from a post-hoc analysis, so they should be considered as hypothesis-generating and confirmed in a prospective study including a large number of patients, especially in the subgroup with PH2, which has been reported to experience neurological deterioration more often. The small number of patients with PH-2 in our study prevented us from obtaining conclusive data for the prediction of this particular type of HT. Second, although the effect of c-Fn was independent of other well-known risk factors related to HT, an increase of plasma c-Fn levels as a result of an acute phase reaction or previous systemic diseases cannot be completely ruled out. However, a direct relationship is likely because increased c-Fn levels were detected at admission before HT in patients in whom radiological findings, biochemical parameters, and vital signs evaluated at the moment the blood samples were taken were not different from those in patients in whom secondary bleeding did not develop, so we cannot attribute the differences in c-Fn levels to a different acute-phase response or a distinct previous comorbidity. Finally, the enzyme-linked immunoabsorbent assay is a slow analytical method, so it would be desirable to develop a faster test to determine c-Fn levels for patients being considered for tPA treatment.

In conclusion, this study demonstrates that high plasma c-Fn levels are independently associated with tPA-induced HT. Further studies including a larger sample of patients are needed to confirm these promising results and to clarify whether c-Fn concentrations may predict PH development in patients treated with t-PA. If this is found to be the case, then c-Fn determination may prove useful in clinical practice to improve the risk/benefit ratio of tPA treatment.

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