Matrix Metalloproteinase Expression Is Related to Hemorrhagic Transformation After Cardioembolic Stroke

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Background and Purpose—In animal models of cerebral ischemia, matrix metalloproteinase (MMP) expression was significantly increased and related to blood-brain barrier disruption, edema formation, and hemorrhagic transformation (HT). MMP inhibitors reduce HT after embolic ischemia in tissue-type plasminogen activator–treated animals. We aimed to determine the relationship between MMPs and HT after human ischemic stroke.

Methods—Serial MMP-2 and MMP-9 determinations were performed by means of ELISA in 39 cardioembolic strokes in the middle cerebral artery territory. Hemorrhagic events were classified according to clinical and CT criteria (hemorrhagic infarction [HI] and parenchymal hematoma [PH]). HT was evaluated on CT at 48 hours (early HT) and again between day 5 and 7 (late HT).

Results—HT was present in 41% of the patients (43.75% early HI, 25% early PH and 31.25% late HI). MMP-2 values were within normal range and were unrelated to HT. Increased expression of MMP-9 (normal range \(97\) ng/mL) was found among patients with and without HT (159.3 \(\pm\) 82 versus 143.9 \(\pm\) 112.6 ng/mL; \(P = 0.64\)). According to HT subtypes, the highest baseline MMP-9 levels corresponded to patients with late HI (240.4 \(\pm\) 111.2 versus 102.5 \(\pm\) 76.7 ng/mL for all other patients, \(P = 0.002\)). Baseline MMP-9 was the only variable associated with late HI in the multiple logistic regression model (OR 9; CI 1.46, 55.24; \(P = 0.010\)). Peak of MMP-9 at the 24-hour time point (250.6 ng/mL) was found before appearance of PH.

Conclusions—MMPs are involved in some subtypes of HT after human cardioembolic stroke. Baseline MMP-9 level predicts late HI and a 24-hour peak precedes early PH. (Stroke. 2001;32:2762-2767.)

Key Words: cerebral ischemia ■ embolism, cerebral ■ hematoma, parenchymal ■ hemorrhagic stroke ■ matrix metalloproteinase

Hemorrhagic transformation (HT) is a feared event that may follow ischemic stroke. Thrombolytic therapy has been shown to be beneficial for acute stroke, although this therapy increases risk of HT.\(^1\) Therefore, identification of the underlying mechanisms of this complication is critical to improvement of the safety profile of thrombolytic agents for stroke treatment.

Matrix metalloproteinases (MMPs) belong to a family of zinc-binding proteolytic enzymes that normally remodel the extracellular matrix.\(^2\) MMP-2 and MMP-9 specifically attack type IV collagen, laminin, and fibronectin, the major components of the basal lamina around cerebral blood vessels.\(^3\) In animal models of cerebral ischemia, MMP expression was increased significantly and related to blood-brain barrier disruption, edema formation, and HT.\(^4,5\) However, whether HT in humans also is related to MMP action remains unknown.

We hypothesize that after MMPs degrade ECM components of the basal lamina, blood elements may extravasate, which leads to HT. In the present exploratory study, we aimed to correlate the expression of MMPs after human cardioembolic stroke with the appearance of different subtypes of HT.

Subjects and Methods

Study Population
From June 1999 through March 2000, we studied prospectively consecutive patients with acute stroke. A total of 110 patients evaluated within the first 12 hours after stroke onset were included in the study. We obtained a detailed history of vascular risk factors from each patient. To identify potential mechanism of cerebral infarction, a set of diagnostic tests was performed that included ECG, chest radiography, carotid ultrasonography, complete blood count and leukocyte differential, and blood biochemistry in all patients; many also underwent special coagulation tests, transthoracic echocardiography, and Holter monitoring. With this information and the neuroimaging data, previously defined etiologic subgroups were determined.\(^6\) Eighty-four (76.4%) patients had a nonlacunar stroke that involved the vascular territory that corresponds to the middle cerebral artery (MCA). Of these, 44 (40%) patients were considered...
to have had a cardioembolic stroke. Most of these patients had atrial fibrillation.

Serial transtemporal Doppler ultrasonographic studies were conducted by use of a Multi-Dop X/TCD device (DWL Elektronische Systeme GmbH) with a hand-held transducer in a range-gated, pulsed-wave mode at a frequency of 2 MHz. During follow-up controls, spontaneous recanalization was diagnosed when a previously absent blood-flow signal reappeared (dampened or normal waveform) for a proximal MCA occlusion (MCAO) or when a previously dampened waveform came within normal range for a distal MCAO.7

We excluded patients with known inflammatory or malignant disease (n=3) and patients with inadequate transtemporal window for transcranial Doppler ultrasound (n=2). A total of 39 patients (51.3% men, mean age 74±15 years) were included in the final analysis. Clinical examination was performed on admission and 12, 24, and 48 hours after symptom onset at time of each MMP determination. Stroke severity was assessed by use of the National Institutes of Health Stroke Scale (NIHSS).

All patients received subcutaneous low-molecular-weight heparin as prophylaxis for deep-vein thrombosis. Intravenous heparin was not administered during the study period, and no patient received tissue plasminogen activator. The present study was approved by the ethics committee of the hospital, and all patients or their relatives gave informed consent.

CT Criteria of HT
On admission, all patients underwent a CT scan within the first 12 hours after stroke onset; the scan was repeated after 48 hours (or earlier when rapid neurological deterioration occurred) to evaluate presence of early HT. In addition, another CT scan was performed to determine factors that could be considered independent predictors of HT subtypes. Cutoff value for MMP-9 with the highest sensitivity and specificity, according to HT groups, was included. Presence and type of HT were defined according to previously published criteria.8,9 Hemorrhagic infarction (HI) was defined as petechial infarction without space-occupying effect, and parenchymal hematoma (PH) was defined as hemorrhage with mass effect. For statistical analysis, both subtypes of HI and PH were considered together, where HI1 indicates small petechiae along the margins of the infarct; HI2, more confluent petechiae within the infarcted area; PH1, hematoma that involves ≥30% of the infarcted area with some slight space-occupying effect; and PH2, hematoma that involved >30% of the infarcted area with substantial mass effect or clot remote to the infarcted area. Symptomatic intracranial hemorrhage was defined as blood at any site in the brain on CT scan and documentation by the investigator of clinical deterioration evidenced by an increase in the NIHSS score of ≥4 points.

MMP-2 and MMP-9 ELISA
Peripheral blood samples were drawn from each patient at study entry and 12, 24, and 48 hours after stroke onset. From the 156 blood draws, we expected extractions, missing data for MMPs included 5 baseline determinations that were so close to the 12-hour time point that only 1 extraction was done. Two missing measurements for the 24-hour and 6 for the 48-hour time points corresponded mostly to patients who died or to technical problems.

EDTA tubes were used to collect the blood. Plasma immediately was separated by centrifugation at 3000 rpm for 15 minutes and stored at −80°C until analysis was done. MMP-2 and MMP-9 levels were determined by commercially available ELISA (Biotrak, Amersham Pharmacia UK). All ELISAs were performed according to manufacturer’s instructions. Our laboratory reference ranges for healthy controls were 41±27.8 ng/mL for MMP-9 (n=62, 58% men, mean age 43 years, and normal range <97 ng/mL) and 630.8±101.8 ng/mL for MMP-2 (n=40, 47% men, mean age 43 years, and normal range 427 to 835 ng/mL). Mean intra-assay coefficients of variation were 8.9% for MMP-9 and 10.7% for MMP-2.

Statistical Analysis
Descriptive and frequency statistical analyses were obtained and comparisons were made by use of the SPSS statistical package, version 9.0. Statistical significance for intergroup differences was assessed by χ² test for categorical variables and Student’s t test and ANOVA for continuous variables. MMP values were distributed normally (Kolmogorov-Smirnov and P-P plot). Baseline or mean MMP levels (mean value of the 4 time points) were used for different analyses.

When indicated, Mann-Whitney U or Kruskal-Wallis test was used, and median and rank are shown. To calculate sensitivity and specificity for MMP values to predict HT, a receiver-operator characteristic curve was configured. Logistic regression analysis was performed to determine factors that could be considered independent predictors of HT subtypes. Cutoff value for MMP-9 with the highest sensitivity and specificity, according to HT groups, was included. P<0.05 was considered statistically significant.

Results
Baseline blood samples, CT scans, and NIHSS scores were obtained within the first 6 hours in 80% of patients and between 6 and 12 hours in all others. HT on CT was detected in 16 (41%) patients. Mean MMP-2 levels for patients with and without HT were included in the normality interval of our laboratory for healthy controls (427 to 835 ng/mL), but mean
MMP-9 levels for patients with and without HT exceeded the reference interval for healthy controls (97 ng/mL; Figure 1). Mean MMP-9 and MMP-2 levels did not differ in terms of presence or absence of HT considered globally (159.3 ± 110.2 vs. 143.9 ± 112.6 ng/mL, P = 0.69 for MMP-9, and 629.1 ± 200.2 vs. 655.1 ± 183.1 ng/mL, P = 0.68 for MMP-2; Figure 1).

On a temporal basis, early HT (occurring at 48 hours) was present in 11 (28.2%) and late HT (detected at the 5- to 7-day CT scan) in 5 patients (12.8%). Figure 2 shows MMP-9 levels for these HT subtypes. MMP-9 levels were higher in the late HT subgroup than in patients with no HT or early HT. Baseline MMP-9 values were 157.6 ± 126.0 for patients without HT, 87.6 ± 65.4 for those with early HT, and 240.4 ± 111.2 ng/mL for those with late HT (P = 0.05).

Table 1 shows main baseline characteristics of HT patients and attending subtypes. In those 16 patients with HT, 7 (43.75%) with early HI, 4 with (25%) early PH, and 5 (31.25%) with late HI were described. Highest MMP-9 values were found in patients with late HI and lowest in those with early HI, and these differences were even higher when baseline MMP-9 levels were considered (Figure 3).

Figure 4 shows temporal profile of MMP-9 according to HT subtypes. Interestingly, the temporal profile of early PH disclosed a peak level of MMP-9 at the 24-hour time point (250.6 ± 165.1 ng/mL). At this time point, all 4 PH patients deteriorated (symptomatic PH). The temporal profile for late HI disclosed a peak baseline level of MMP-9. This time point offered the best association with development of late HI. Baseline MMP-9 level was statistically higher for patients with late HI than for the remaining patients (240.4 ± 111.2 versus 102.5 ± 76.7 ng/mL, P = 0.002). These differences also were observed for mean MMP-9 level (212.4 ± 89.0 ng/mL for late HI versus 130.3 ± 80.9 ng/mL for all other patients; P = 0.049). At the 24-hour time point, MMP-2 levels were also higher if late HI occurred (721.0 ± 217.8 ng/mL for late HI versus 537.6 ± 116.1 ng/mL for all other patients; P = 0.009).

Table 2 illustrates different subtypes of HT according to time of spontaneous recanalization. A different recanalization pattern was found between early and late HI (P = 0.036). No patient who recanalized during the first 12 hours after symptom onset (early recanalization) had late HT. Conversely, 6 of 7 (85.7%) patients who recanalized in the first 12 hours experienced early HI.

To confirm the association between MMP-9 and late HI, a multiple logistic regression model was used (Table 3). Although hypertension and absence of early recanalization were associated significantly with late HI on univariate analysis, only baseline MMP-9 levels (OR 9; CI 1.46, 55.24; P = 0.010) remained as an independent predictor of late HI. A cut point

| TABLE 1. Main Baseline Characteristics of Patients According to Presence and Subtype of HT |
|---------------------------------|-----------------|-----------------|-----------------|-------|
|                                | Early HI (n=7)  | Early PH (n=4)  | Late HI (n=5)   | P     |
| Men                            | 3 (42.8%)       | 3 (75.0%)       | 2 (40)          | 0.511 |
| Age, y                         | 77.0            | 72.0            | 83.0            | 0.288 |
| Hypertension                   | 2 (28.5%)       | 4 (100)         | 5 (100)         | 0.009*|
| Hyperlipidemia                 | 0 (0)           | 0 (0)           | 2 (40)          | 0.081 |
| Diabetes                       | 0 (0)           | 2 (50)          | 2 (40)          | 0.118 |
| Previous stroke                | 1 (14.2%)       | 0 (0)           | 2 (40)          | 0.287 |
| Temperature, °C                | 36.6            | 36.3            | 36.0            | 0.918 |
| NIHSS score                    | 20              | 20              | 20              | 0.986 |
| Early recanalization           | 6 (85.7%)       | 1 (25.0)        | 0 (0)           | 0.014*|
| Infarct volume, cm³            | 60.6            | 133.1           | 22.0            | 0.417 |
| Systolic blood pressure, mm Hg | 135.0           | 170.0           | 180.0           | 0.173 |
| Diastolic blood pressure, mm Hg| 70.0            | 87.5            | 100.0           | 0.026*|
| Glucose, mg/dL                 | 116.0           | 360.0           | 159.0           | 0.026*|
| Platelets                      | 178,000         | 179,000         | 188,000         | 0.685 |
| Leukocytes, ×10³/L             | 9700            | 9700            | 7600            | 0.331 |
| Partial thromboplastin time    | 32.0            | 32.6            | 32.1            | 0.018*|
| Fibrinogen, g/L                | 3.8             | 4.0             | 4.2             | 0.847 |
| MMP-2, ng/mL                   | 647.6           | 636.2           | 825.1           | 0.587 |
| MMP-9, ng/mL                   | 70.7            | 81.0            | 223.8           | 0.047*|

Values are median or n (%). Baseline data are shown, except for early recanalization assessed within 12 hours of symptom onset and infarct volume measured at 48-hour CT scan.

*Factors with a value of P < 0.05.
for MMP-9 of 144.81 ng/mL had sensitivity 80% and specificity 79.2% to detect presence of late HI.

HT was deep in the MCA territory in 10 patients (25.6%) and involved the superficial MCA territory in the 6 remaining (15.4%). MMP expression was similar regardless of whether HT was located deep in the MCA territory or involved the superficial MCA territory. Although mean MMP-9 level was higher in superficial (178.7±119.4 ng/mL) than in deep HT (147.6±56.0 ng/mL) and, conversely, mean MMP-2 level was higher in deep HT (681.7±218.4 ng/mL) than in superficial HT (541.5±140.2 ng/mL), none of these differences achieved statistical significance (P=0.76 and P=0.33, respectively).

Discussion

The present study shows for the first time an association between MMP-9 expression and several subtypes of HT after human cardioembolic stroke. In a large CT study of acute cerebral embolism, Okada et al10 noted a spectrum of intracranial bleeding in 40.7% of patients not receiving anticoagulants, including 8.6% of PHs. These numbers are similar to the HT rates in our series (41% and 10.2%, respectively).

In the present study, MMP-9 especially was increased in cases of late HI. Also, a significant increase in latent MMP-9 was found in baboons with HT.5 In fact, baseline MMP-9 level was the only factor independently associated with late HI among our patients.

Our findings support the idea that blood plasma or cellular elements may extravasate after degradation of ECM components of the basal lamina by MMPs. After experimental MCAO, a gradual loss of laminin-1, laminin-5, fibronectin, and collagen IV antigens within the basal lamina occurs.11,12 Hamman et al13 demonstrated a significant correlation between development of HT and regional loss of basal lamina after 3 hours of MCAO.

MMP-2 and MMP-9 released from vascular endothelium and leukocytes during the inflammatory phase of ischemic stroke use collagen IV and laminin as substrates.14,15 Ogata et al16 found that leukocyte plugging in microvessels of brain tissue was more frequent in hemorrhagic infarcts than in pale infarcts. Neutrophils also are known to use MMPs for their migration.17

After intracerebral injection of MMP-2, brain regions displayed necrosis, hemorrhage, and migration of blood cells to the site of the injury.18 In the present study, MMP-2 was related only to late HI at the 24-hour time point, and other studies did not find constitutive expressed MMP-2 to increase the risk of HT.5

Classic works have stated that the primary mechanism for HT in cardioembolic stroke appears to be reperfusion of infarcted or ischemic brain tissue after distal migration or dissolution of the embolus.19 Occasionally, delayed hemorrhage occurs without reperfusion, and in these cases, the source of the hemorrhage is postulated to be collateral.
circulation to the damaged area. An interesting association also occurs between time to artery reopening and presence of HT in our series. Most patients with early HI recanalized within 12 hours, but all patients who developed late HI had an absence of early spontaneous recanalization.

In a previous study, MMP expression was correlated strongly with extent and duration of MCAO. Taken together, these data make it possible to hypothesize that the longer the MCAO, the higher the MMP-9 expression with a greater basal lamina disruption responsible for the HT, after restoration of circulation to the injured capillary bed either by reopening of the initial site of occlusion or by establishment of collateral circulation.

Several conditions that are aggravated by advancing age, such as long-term hypertension and diabetes, target the microvasculature and may contribute to loss of microvascular integrity. All PH and late HI patients in our series were hypertensives, and many were diabetics. MMP-9 proteolytic activity easily could undermine the integrity of a weakened arterial wall.

We considered the possibility that MMPs played an active role in the matrix degradation that either initiated or propagated the formation of PHs. In the present study, an MMP-9 peak was found at the 24-hour time point before the presence of a symptomatic PH. Rupture of the vascular wall is likely in several instances of intrafarkt hematoma. In the present study, determination of whether this high MMP-9 peak is the cause of the hematoma or simply represents a marker of the extent of tissue destruction after PH was not possible, because the cause-effect relationships between HT and the biochemical marker were not established.

### Table 2. Relation Between HT and MCA Status

<table>
<thead>
<tr>
<th>HT Subtype</th>
<th>Early Recanalization (&lt;12 h), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non HT</td>
<td>Yes 11 (50)</td>
</tr>
<tr>
<td>Early HI</td>
<td>Yes 6 (85.7)</td>
</tr>
<tr>
<td>Early PH</td>
<td>Yes 1 (25)</td>
</tr>
<tr>
<td>Late HI</td>
<td>Yes 0 (0)</td>
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</tbody>
</table>

In the fifth case of late HI, only 1 TCD recording was available until 12 hours; hence, recanalization could not be assessed. HT subtypes showed a different distribution (P=0.036) mainly due to the opposite recanalization pattern between early and late HI.

### Table 3. Factors Associated With Late Hemorrhagic Infarctions on Univariate Analysis and Multiple Logistic Regression Model

<table>
<thead>
<tr>
<th>Late HI</th>
<th>Univariate Analysis, OR (95% CI)</th>
<th>Logistic Regression, OR (95% CI)</th>
<th>logLR, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline MMP-9</td>
<td>15.2 (1.37, 167.98)</td>
<td>9 (1.46, 55.24)</td>
<td>0.010</td>
</tr>
<tr>
<td>Absence of early recanalization</td>
<td>0.06 (0.003, 1.36)</td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>13.6 (0.68, 271.03)</td>
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MMP-9 cut point level (144.81 ng/mL) was obtained from receiver-operator characteristic curve.

Our series included cardioembolic stroke patients, and many of them need anticoagulant therapy as secondary prevention. We suggest that baseline MMP-9 levels may help to decide the safest moment to initiate anticoagulation.

Because plasmin is involved in the cascade that processes proMMP-9 to the active form, administration of tissue plasminogen activator may activate and promote the destructive potential of this enzyme, thereby leading to hemorrhage. This hypothesis could explain the higher rates of HT after thrombolysis and the reduction of tissue plasminogen activator–induced hemorrhages after administration of an MMP inhibitor (BB-94) in a rabbit cardioembolic stroke model.

Measurement of plasma levels of MMPs to identify patients at higher risk of HT could prove useful, and its potential usefulness should be tested in a future clinical trial.

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The online version of this article, along with updated information and services, is located on the World Wide Web at:
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