A Matrix Metalloproteinase Protein Array Reveals a Strong Relation Between MMP-9 and MMP-13 With Diffusion-Weighted Image Lesion Increase in Human Stroke

Anna Rosell, MSc; José Alvarez-Sabín, MD, PhD; Juan F. Arenillas, MD, PhD; Alex Rovira, MD; Pilar Delgado, MD; Israel Fernández-Cadenas, MSc; Anna Penalba; Carlos A. Molina, MD, PhD; Joan Montaner, MD, PhD

Background and Purpose—Matrix metalloproteinases (MMPs) are involved in tissue destruction produced by the neuroinflammatory response that follows ischemic stroke. In the present study we use an MMP array to investigate the blood levels of several MMPs in stroke patients and its relation with brain tissue damage and neurological outcome.

Methods—Twenty-four patients with middle cerebral artery occlusion who received thrombolytic therapy were included. Blood samples were drawn before tissue plasminogen activator treatment and an MMP array (multiplex enzyme-linked immunosorbent assay [ELISA]) was performed including gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, MMP-8, and MMP-13), stromelysines (MMP-3 and MMP-10), and MMP endogen inhibitors (TIMP-1 and TIMP-2). To assess tissue lesion a serial multimodal MRI study was performed (pretreatment and at 24 hours).

Results—Neither initial diffusion lesion nor hypoperfused volume was associated with metalloproteinase expression within the first 3 hours after stroke onset. Nevertheless, a strong correlation was found between MMP-9 and MMP-13 with diffusion-weighted image (DWI) lesion expansion ($r = 0.54$, $P = 0.05$ and $r = 0.60$, $P = 0.017$, respectively). Baseline levels of both MMP-9 (OR, 14; 95% CI, 1.5 to 131; $P = 0.019$) and MMP-13 (OR, 73; 95% CI, 3.9 to 1388; $P = 0.004$) were independent predictors of final increase in brain infarct volume at 24 hours.

Conclusions—Our results demonstrate that within the neuroinflammatory response, high levels of MMP-9 and MMP-13 are involved in DWI lesion growth despite thrombolytic therapy, suggesting its ultra-early role in brain injury. (Stroke. 2005;36:1415-1420.)

Key Words: fibrinolysis • metalloproteinase • magnetic resonance imaging, diffusion-weighted • stroke • tissue plasminogen activator

Ischemic stroke activates a complex cascade of events,1 some of which occurs within the first minutes after stroke onset, such as glutamate release, and others, as leukocyte infiltration and cerebral edema, which are observed hours or days after brain vessel occlusion. Matrix metalloproteinases (MMPs) constitute a large family of zinc-dependent endoproteases involved in the degradation of extracellular matrix components that play an important role in several steps of the molecular cascade following stroke.2,3

Expression of some metalloproteinases such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B) is upregulated after cerebral ischemia4–7 and contributes to infarct extent, blood–brain barrier breakdown, and poor neurological outcome.8–10 We have previously shown high MMP-9 level after acute ischemic stroke related to neurological outcome11 and hemorrhagic transformation in patients who received thrombolytic treatment.12 Others have demonstrated that hypothermia reduces MMP-9 expression, whereas thrombolytic treatment activates MMP-9,13 suggesting clinical benefit from combination therapies targeting MMPs, as has been demonstrated in animal models of cerebral ischemia.14–16

Regarding human stroke, a recent investigation showed that MMP-9 is a good predictor of infarct volume measured as a diffusion-weighted image (DWI) lesion when evaluated within 6 hours of symptom onset.17

To obtain further knowledge of mechanisms that could interfere with successful thrombolytic therapy, this study attempts to evaluate the ultra-early role of MMPs after hyperacute stroke in different areas of ischemic brain (diffusion and perfusion abnormalities) by studying new members of this family in shorter time periods. We hypothesize that some MMPs might be involved in tissue damage within the first 3 hours after stroke onset and therefore might be clinically relevant for neurological outcome following standard thrombolytic therapy.
Materials and Methods

Study Population and Clinical Protocol

Our target was stroke patients involving the middle cerebral artery (MCA) territory who received a multimodal MRI study before thrombolytic treatment. The study included 65 consecutive patients with an acute stroke admitted to the emergency department who received thrombolytic therapy in a standard 0.9 mg/kg dose (10% bolus, 90% continuous infusion for 1 hour) within 3 hours of symptom onset. Five had a basilar artery occlusion, 2 had a posterior cerebral artery occlusion, and 58 had a MCA occlusion, all of them documented on transcranial Doppler. Within the 58 MCA occlusions, 25 underwent a MRI study within the first 3 hours of stroke onset. The remaining patients did not receive the MRI study because of the lack of availability of the technique 24 hours per day or because of any contraindication to perform MRI study. A control DWI was repeated after 24 hours to evaluate lesion increase. All patients with a known inflammatory or malignant disease were excluded. Finally, 24 patients with a MCA occlusion received the baseline study protocol including a complete MRI study before tissue plasminogen activator treatment.

A detailed history of vascular risk factors was obtained from each patient. To identify potential mechanisms of cerebral infarction, all patients underwent a set of diagnostic tests (including electrocardiogram, chest radiography, carotid ultrasonography, complete blood count, leukocyte differential, and blood biochemistry). Clinical examination was performed on admission and at 12, 24, and 48 hours from symptom onset. Stroke severity and neurological outcome were assessed using the National Institutes of Health Stroke Scale (NIHSS). Transcranial Doppler measurements were performed by an experienced neurologist using a Multi-Dop X4 (DWL Elektronische Systeme GmbH, Sipplingen, Germany) device, with a hand-held transducer in a range-gated, pulsed-wave mode at a frequency of 2 MHz. Proximal MCA occlusion was defined as the absence of flow or the presence of minimal flow signal throughout the MCA, accompanied by flow diversion in the ipsilateral anterior cerebral artery and posterior cerebral artery. Distal MCA occlusion was defined as a diffuse dampening of the mean blood flow velocity in the affected MCA >21% compared with the contralateral MCA. This study was approved by the ethics committee of the hospital and all patients or relatives gave informed consent.

MRI Protocol

All MRI studies were performed with a 1.5-T whole-body imaging system with 24-mT/m gradient strength, 300-ms increase time, and an echo-planar–capable receiver equipped with a gradient overdrive (Magnetom Vision Plus, Siemens Medical Systems, Germany). The images included axial T2-weighted susceptibility-based echo-planar gradient-echo sequence (0.8/29/1 [TR/TE/acquisitions]; total acquisition time 2 seconds); axial diffusion-weighted echo-planar spin-echo sequence (4000/100/2 [TR/TE/acquisitions]; total acquisition time 56 seconds); and axial perfusion-weighted echo-planar gradient-echo sequence (2000/60/40 [TR/TE/acquisitions]; total acquisition time 80 seconds).

DWI was obtained with a single-shot spin-echo echo-planar pulse sequence with diffusion gradient b-values of 0, 500, and 1000 s/mm² along all 3 orthogonal axes over 15 axial sections, with 5-mm slice thickness (interslice gap of 1.5 mm), a field of view of 230 mm, and 96×128 matrix. The acquisition time for the DWI equaled 56 seconds. Perfusion-weighted image (PWI) was acquired by using a bolus of gadolinium-based contrast material (Magnevist; Schering AG, Berlin, Germany) for selected 13- to 15-section positions measured 40 times sequentially. The perfusion-weighted sequence generated a time-to-peak (TTP) map for each section position that was immediately available for interpretation at the console with all the other images. PWI was obtained using 5-mm-thick sections, an interslice gap of 1.5 mm, a field of view of 240 mm, and 128×128 matrix. In all patients, baseline MRI study was fully completed. Only 21 patients received the second DWI examination at 24 hours, and the remaining patients were severely impaired and were unable to tolerate this second examination.

Volumetric Assessment of Lesion Size

Volume measurements of the extent of tissue abnormality on DWI and on TTP maps were performed by an experienced neuroradiologist, blinded to clinical and laboratory data, using a manual tracing technique. The perimeter of the area of abnormal high-signal intensity was traced on each DWI and TTP map. All measured areas were multiplied by the slice distance to obtain the total lesion volumes for both the DWI and TTP maps (cubic centimeters [cc]). The extent of ischemic penumbra was calculated as the difference between baseline PWI and DWI volumes, and the increase in DWI lesion was assessed as the difference between final DWI and initial DWI, divided per initial DWI and expressed as a percentage (%).

Multiplexed MMP Array

Peripheral blood samples were drawn from each patient at study entry (before tissue plasminogen activator administration). 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. SearchLight Human MMP Array 1 (Pierce, Rockford, Ill) was used to measure MMPs; this assay consists of multiplexed sandwich enzyme-linked immunosorbent assay for the quantitative measurement of 9 proteins in each sample: gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, MMP-8, and MMP-13), stromelysines (MMP-3 and MMP-10), and endogen inhibitors (TIMP-1 and TIMP-2). Figure 1A. Each sample was assayed 2 times and the mean value of both measurements was used. The mean intra assay coefficients of variation were <15% for all biomarkers measured.

The enzyme-substrate reaction produces a chemiluminescent signal detected with a cooled CCD camera (Pierce). The images were analyzed by ArrayVision version 8.0 software (Imaging Research). Although the array standard curves are given in pg/mL units, we have converted them to ng/mL for easier interpretation.

Statistical Analyses

Descriptive and frequency statistical analyses were obtained and compared using SPSS 12.0. MMP levels did not have a normal distribution (Kolmogorov–Smirnov and P-P plot); therefore, values are expressed as median (interquartile range). Statistical significance for intergroup differences was assessed by the Fisher exact test (for categorical variables) and the Mann–Whitney U or Kruskal–Wallis
tests (for continuous variables). The Spearman coefficient was used to study correlations between continuous variables. Significant probability values for multiple comparisons were adjusted using Bonferroni correction. Receiver–operator curves were obtained for MMPs to identify the best cutoff points for predicting different DWI lesion increases. Finally, a multiple logistic regression model was performed to detect independent markers of DWI lesion increase. P/H110210.05 was considered statistically significant.

**Results**

**Patients Characteristics and MRI Study**

Mean age of the study group (54% men) was 72±14 years; the main baseline characteristics, including risk factors and other clinical variables, are shown in Table 1. The planimetric measurement showed an initial DWI volume of 9.8 cc (5.4 to 17.6), PWI volume of 180.5 cc (110.1 to 205.5), and a calculated volume of ischemic penumbra of 153 cc (98–198). Control MRI performed 24 hours later found a DWI volume of 36.4 cc (16.3 to 85.9), with a median percentage of increase of 173% (23–684).

No correlation was found between initial DWI lesion or initial PWI volume and any of the risk factors or clinical variables shown in Table 1. Among clinical variables, only baseline NIHSS score and presence of proximal or distal occlusion were weakly related to an increase in DWI volume at 24 hours (r=0.38, P=0.08, and 277% versus 14% P=0.06, respectively). The MMP array measurements and results are shown in Figure 1 and Table 2.

**Relationship Between MMP Levels and Extent of Brain Lesion**

All measured biomarkers were similar in terms of the presence of a proximal (75%) or distal MCA occlusion (data not shown). No correlation existed between baseline biomarkers and DWI volumes at arrival. Moreover, no correlation was found between protein levels and hypoperfused tissue volume (PWI) at baseline MRI study or ischemic penumbra. Among the 9 metalloproteinases assessed by MMP array, only one gelatinase and one collagenase were strongly correlated with an increase in the extent of DWI lesion during the first 24 hours (Figure 2). Both MMP-9 (gelatinase B) and MMP-13 (collagenase-3) baseline levels were positively correlated with an increase in DWI lesion (r=0.54, P=0.05 and r=0.60, P=0.017, respectively). A positive correlation was also found between these 2 metalloproteinases (r=0.59, P=0.003).

To better explore this finding, patients were divided into 2 groups according to increase in DWI volumes. Half of the study patients had an increase in DWI volume >180% at 24 hours. Multivariate analyses to assess the main factors related to large increments (>180%) in DWI lesion were performed, with no relationship found between lesion growth at 24 hours and any risk factor or clinical variable evaluated. Regarding

**TABLE 1. Baseline Characteristics of the Stroke Population Studied**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>72.1±14.5</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>13 (54.2%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (33.3%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>8 (33.3%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4 (16.7%)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>9 (37.5%)</td>
</tr>
<tr>
<td>Coronary disease</td>
<td>0</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>7 (29.2%)</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>3 (12.5%)</td>
</tr>
<tr>
<td>SBP</td>
<td>153.8±23.2</td>
</tr>
<tr>
<td>DBP</td>
<td>81.7±10.5</td>
</tr>
<tr>
<td>Glycemia</td>
<td>138.6±71.5</td>
</tr>
<tr>
<td>Proximal occlusion</td>
<td>18 (75%)</td>
</tr>
<tr>
<td>Baseline NIHSS score</td>
<td>14.5 (8.5–20.75)</td>
</tr>
</tbody>
</table>

DBP indicates diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; SBP, systolic blood pressure.

**TABLE 2. MMP Array Values**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Values, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>8.55 (5.13–12)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>200.6 (116.7–303.4)</td>
</tr>
<tr>
<td>MMP-3</td>
<td>4.37 (2.72–6.06)</td>
</tr>
<tr>
<td>MMP-8</td>
<td>0.96 (0.96–0.96)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>92.34 (55.69–265.53)</td>
</tr>
<tr>
<td>MMP-10</td>
<td>0.38 (0.30–0.44)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>3.08 (1.76–5.24)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>57.13 (37.71–75.04)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>77.77 (62.82–99.52)</td>
</tr>
</tbody>
</table>

Results expressed as median (interquartile range).

Figure 2. Extent of damaged tissue (DWI volume increase) is correlated with MMP-9 (r=0.54, P=0.05) and MMP-13 levels (r=0.60, P=0.017).
by guest on December 30, 2017

Stroke July 2005

1418

crovessels after cerebral ischemia causes parenchyma de-
MMP-induced degradation of basal lamina surrounding mi-

no MMP level was related to initial neurological state before

of patients with MMP-13 concentrations >3 ng/mL had DWI
increases >180% (P=0.023 and P=0.001, respectively). In
fact, these cutoff points yielded 80% sensitivity and 81.8%
specificity for MMP-9 and 90% sensitivity and 91% specific-
ity for MMP-13 to predict DWI increases >180%

Furthermore, both biomarkers were independent predictors
of an increase >180% of the extent of DWI lesion at 24
hours, even when other classical baseline factors such as
stroke severity (assessed as NIHSS score) and the presence
of a proximal occlusion (assessed by transcranial Doppler)
were included in the regression model. Odds ratios were 14 for
MMP-9 (1.5 to 131; P=0.019) and 73 for MMP-13 (3.9 to
1388; P=0.004).

Metalloproteinases and Neurological Outcome

No MMP level was related to initial neurological state before
tissue plasminogen activator treatment, but several significant
associations with neurological outcome appeared later at
different time points.

Clinical assessment revealed that only both metalloprotein-
ases related to lesion growth were weakly associated with
poor neurological state, as reflected by MMP-9 correlation
to stroke severity (NIHSS 24 hours: r=0.52, P=0.04) and with
NIHSS score increase at 48 hours (MMP-9: r=0.46, P=0.08
and MMP-13: r=0.45, P=0.11). Among the remaining
MMPs assessed, no other correlations were found. Regarding
MMP inhibitors achieved, both TIMP-1 (NIHSS increase at
48 hours: r=0.69, P=0.0004) and TIMP-2 (NIHSS increase
at 48 hours: r=0.56, P=0.021) were related to neurological
outcome.

Discussion

MMP-induced degradation of basal lamina surrounding mi-
cro vessels after cerebral ischemia causes parenchyma de-
struction related to CT scan-measured infarct volume and
hemorrhagic transformation.11,12 New MRI techniques such as
DWI and PWI make it possible to distinguish between
different tissue brain areas, providing valuable information
for correlation studies to recognize new potential biomarkers
responsible for cell damage (DWI) or low cerebral blood flow
(PWI).17–19 Altogether, our results reveal that patients who
receive thrombolytic therapy show pretreatment expression
of MMP-9 and MMP-13 related to ulterior tissue destruction
within the following 24 hours, reflected as an increase in the
extent of DWI lesion.

Clear evidence from animal models has recently shown
that the extent of brain infarction is partially caused by MMP
activity that attacks different components of extracellular
matrix and specifically by MMP-9 that has been reported to be
overexpressed and tissue located.16,18,20–22 Therapeutic
assays in rodents with MMP-9 inhibitors15,23 and MMP-9
gene knockouts15,24 have demonstrated infarct size reduction
and block blood–brain barrier disruption.

Contrary to the well-established deleterious role of
MMP-9, this is the first time to our knowledge that colla-
genase-3 (MMP-13) has been shown to be involved in tissue
injury after stroke. This metalloproteinase has been thor-
oughly studied in aggressive cancer as a biomarker of tumor
progression,25 in bone morphogenesis26 (in which it is in-
volved in bone development and remodeling), and in abdom-
inal aortic dilatation and rupture.27 Only one earlier study has
attempted to measure MMP-13 in human stroke within the
first 12 hours of symptoms as compared with a healthy
control group, but no difference was found.13

Our study provides new information about MMP-13 in a
shorter time period, reporting higher protein values than
others did in a wider time range after stroke onset,13 and it
also describes its relation with ischemic brain tissue. In fact,
extracellular matrix degradation after cerebral ischemia might
be, in part, caused by collagen degradation by secreted
enzymes like MMP-13, because type IV collagens are its
specific substrates and this basal lamina component is lost
after cerebral ischemia.28 Cell-type source of MMP-13 after
stroke remains to be investigated. Because MMP-9 and
MMP-13 were correlated in our stroke population, activation
pathways between both MMPs are possible.29
No correlation was found between baseline DWI lesion and any metalloproteinase within first 3 hours after stroke symptoms, ruling out a simplistic acute phase-reactant explanation. Interestingly, correlations with DWI lesion increase 1 day later would suggest that endogen expression of some MMPs is implicated in brain tissue destruction.

Similarly, we found no correlation between any MMP and baseline NIHSS scores, but neurological outcome was later positively related with MMP-9. We hypothesize that initial metalloproteinase expression is not caused by stroke severity, but that baseline expression of some MMPs is in part responsible for neurological impairment, as reported by other studies. In fact, baseline interindividual differences in plasmatic levels of this metalloproteinases after ischemic injury imply a different response in the increase of DWI lesion extent and in neurological evolution despite thrombolytic therapy. Some reports regarding genetic background caused variation in tissue plasminogen activator on the levels of those biomarkers. These results also suggest that both metalloproteinases are involved in tissue injury and cell death, counteracting the benefits of thrombolytic therapy.

Acknowledgments
A.R. is the recipient of a predoctoral grant from the Institut de Recerca, Hospital Vall d’Hebron. We are grateful to Manolo Quintana for statistical advice and to Toni Lozano and Ineabel Carrillo for their technical assistance.

References


A Matrix Metalloproteinase Protein Array Reveals a Strong Relation Between MMP-9 and MMP-13 With Diffusion-Weighted Image Lesion Increase in Human Stroke
Anna Rosell, José Alvarez-Sabín, Juan F. Arenillas, Alex Rovira, Pilar Delgado, Israel Fernández-Cadenas, Anna Penalba, Carlos A. Molina and Joan Montaner

*Stroke*. 2005;36:1415-1420; originally published online June 9, 2005;
doi: 10.1161/01.STR.0000170641.01047.cc

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/36/7/1415

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/