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

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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-ml/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-nT/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.
baseline biomarkers, we found higher plasma levels of both MMP-9 and MMP-13 among those patients with DWI volume increases >180% at follow-up (208 versus 53 ng/mL, \( P=0.034 \) and 5.8 versus 1.8 ng/mL, \( P=0.008 \), respectively; Figure 3).

The receiver–operator curves identified MMP-9 >100 ng/mL and MMP-13 >3 ng/mL as the best cutoff points to predict large DWI increases (>180%). Using these cutoffs, 77.8% of patients with MMP-9 levels >100 ng/mL and 89% 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% specificity 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 metalloproteinases related to lesion growth were weakly associated with poor neurological state, as reflected by MMP-9 correlation to stroke severity (NIHSS 24 hours; \( r=0.52, P=0.004 \)) 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 microvessels after cerebral ischemia causes parenchyma destruction 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 inhibitors\(^ {15,23} \) and MMP-9 gene knockouts\(^ {15,24} \) 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 collagenase-3 (MMP-13) has been shown to be involved in tissue injury after stroke. This metalloproteinase has been thoroughly studied in aggressive cancer as a biomarker of tumor progression,\(^ {25} \) in bone morphogenesis\(^ {26} \) (in which it is involved in bone development and remodeling), and in-abdominal 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 endogenous 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 by presence of functional polymorphisms, which could influence MMPs level, might partially explain these differences.

Finally, tissue inhibitors of matrix metalloproteinases (TIMP) findings are somewhat intriguing, because we expected a protective role for these MMPs inhibitors; however, the antibody used may recognize both free and complexed MMP-TIMP forms, making it difficult to give an explanation for our results.

Therefore, our results support the fact that biochemical data may add information to the modern neuroimaging techniques and altogether might help to better guide stroke thrombolysis in the future. Because we focused our research on identifying baseline biomarkers (pretreatment) of tissue damage, we did not consider other factors occurring at later time points (eg, MCA recanalization) that are well-known factors of infarct extension.

The main limitation of the present study is the small size of our study group and we have to be cautious about the predictive role of both biomarkers; however, strict criteria were used to select cardiomebolic strokes with MCA occlusions that received tissue plasminogen activator and complete MRI study within the first 3 hours after stroke onset. Moreover, the assessment of changes in lesion size could be biased, because 3 severe patients in whom the second MRI was not performed might have a different profile of lesion size change than those who were well enough at 24 hours for the control MRI. However, biomarker prediction of DWI lesion increase could have improved if we could have studied those patients with severe strokes in whom an infarct increase was expected because they also had high MMP-9 and MMP-13 levels. Furthermore, a control group of healthy patients and repeated measurement of the studied biomarkers might improve the quality of a future study, because it would provide valuable information on the degree of expression of the MMPs studied in normal plasma and of the effect of tissue plasminogen activator on the levels of those biomarkers.

In conclusion, we have demonstrated that in the hyperacute phase of stroke, MMP-9 and MMP-13 expression is related to an increase in DWI lesion within the first 24 hours. Our 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.

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

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