MMP-9–Positive Neutrophil Infiltration Is Associated to Blood–Brain Barrier Breakdown and Basal Lamina Type IV Collagen Degradation During Hemorrhagic Transformation After Human Ischemic Stroke

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Background and Purpose—An abnormal expression of some matrix metalloproteinases (MMPs) is related with hemorrhagic transformation events after stroke. Our aim was to investigate MMP-2 and MMP-9 in the ischemic brain and its relation with blood–brain barrier breakdown after hemorrhagic transformation in human stroke.

Methods—We assessed 5 cases of fatal ischemic strokes with hemorrhagic complications; brain samples were obtained from infarct, hemorrhagic, and contralateral tissue. MMP-9 and MMP-2 content was analyzed by zymography and immunohistochemistry was performed to localize MMP-9 and to assess collagen IV integrity in the basal lamina. Laser capture microdissection was performed to isolate blood–brain barrier vessels to study these MMPs.

Results—Overall, MMP-9 levels were higher both in hemorrhagic and nonhemorrhagic infarcted tissue compared to contralateral areas (P<0.0001 and P<0.05). Moreover, levels of the cleaved MMP-9 85kDa-form were significantly elevated in the hemorrhagic compared to nonhemorrhagic and contralateral areas (P=0.033 and P<0.0001). No changes were found for MMP-2 content. Immunostaining revealed a strong MMP-9–positive neutrophil infiltration surrounding brain microvessels associated with severe basal lamina type IV collagen degradation and blood extravasation. Microdissection confirmed that content of MMP-9 was similarly high in microvessel endothelium from hemorrhagic and infarcted areas compared to contralateral hemisphere vessels (P<0.05), pointing to neutrophils surrounding dissected microvessels as the main source of MMP-9 in hemorrhagic areas.

Conclusions—Our results show a strong neutrophil infiltration in the infarcted and hemorrhagic areas with local high MMP-9 content closely related to basal lamina collagen IV degradation and blood–brain barrier breakdown. Microvessel and inflammatory MMP-9 response are associated with hemorrhagic complications after stroke. (Stroke. 2008;39:1121-1126.)

Key Words: blood–brain barrier ▪ collagen IV ▪ hemorrhagic transformation ▪ MMP-9 ▪ neutrophil ▪ stroke

Ischemic stroke is a leading cause of death and disability worldwide that occurs mainly because of occlusion of a cerebral artery by a thrombus. Unless there is an early restoration of blood flow, the brain parenchyma can suffer irreversible damage because of neuronal death, blood–brain barrier (BBB) breakdown, and cellular edema.

The BBB protects the brain against molecules and pathogens circulating through the blood stream. This specialized neurovascular interface functionally comprises the endothelial tight junction, basal lamina, and astrocytic end feet. BBB leakage and the extravasation of blood constituents to the brain parenchyma are highly correlated with loss of basal lamina antigens.1 Thus, maintaining basal lamina integrity is a key challenge to prevent hemorrhagic complications and brain damage induced by this phenomenon.

Recently, there has been an emphasis on investigating the role of matrix metalloproteinases (MMP) which can degrade almost all components of the extracellular matrix and basal lamina such as laminins, fibronectin, or type IV collagen, weakening brain microvessels and predisposing them to rupture and increasing the risk of cerebral hemorrhage.2,3 These previous investigations demonstrated that an abnormal expression of MMP-2 (gelatinase A) or MMP-9 (gelatinase B) appears after cerebral ischemia4–6 and in lipopolysaccharide-injured brains,7 contributing to brain injury and BBB breakdown. Pharmacological or genetic inhibition of MMP-9

Received August 3, 2007; final revision received September 3, 2007; accepted September 19, 2007.
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Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.107.500868
significantly decreases infarct size and the risk of hemor-
ragic complications,\textsuperscript{8,9} whereas tissue plasminogen activa-
tor-associated hemorrhage and edema appear to be correlated to
MMP-9 dysregulation.\textsuperscript{10,11}

In humans, investigations in peripheral blood have shown high
MMP-9 levels in patients with ischemic stroke and, more importantly, that MMP-9 levels are related to poor neurological outcome, infarct growth, and hemorrhagic transformation (HT) events.\textsuperscript{12,13} We recently reported evidence that neutrophil brain recruitment occurs in the perivascular ischemic areas after human stroke.\textsuperscript{14} However, the precise relationship between dysregulated MMP, inflammatory cell responses, and the concomitant hemorrhagic conversion in stroke remains to be fully elucidated.

In the present study we aimed to investigate in human brain samples, the relationships between MMP-9 protein expres-
sion, basal lamina degradation, inflammatory neutrophil infiltr-
ation, and the presence of hemorrhage after ischemic stroke.

**Materials and Methods**

**Brain Tissue Samples**

Five deceased patients who had an ischemic stroke within the previous 4 days were included in the study (3 women and 2 men). All selected cases had a hemorrhagic transformation of the infarcted area. One case received thrombolytic treatment 3 days before death and all other cases experienced spontaneous hemorrhagic transforma-
tions. On autopsy and during macroscopic examination, infarcted area was delimited by an experienced neuropathologist (mainly through consistence and color of the parenchyma) and neuroradiology images were used to help in obtaining brain tissue from ipsilateral (infarct [I] or HT) and contralateral (C) hemisphere. All samples were obtained within the first 6 hours after death and snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) for gelatin zymographies, or fixed with formalin for immunohistochemistry techniques. This study was approved by the Ethics Committee of the hospital and informed consent was acquired from all relatives before the autopsy. Frozen samples were homogenated for gelatin zymography. Brain tissue (0.2 g) was mixed with 0.7 mL of cold lysis buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mM NaCl, 5 mmol/L CaCl\(_2\), 0.05% Brij-35, 0.02% NaN\(_3\), and 1% Triton X-100) containing protease inhibitors (phenylmethanesulphonylfluoride [PMSF] and aprotinin) and gelatinase recombinant proteins including MMP-2 and MMP-9 (Chemicon) were also performed with samples. After electrophoresis, gels were washed to remove SDS with 2.5% Triton X-100 for 1 hour and incubated at 37°C with incubation buffer containing (50 mmol/L Tris-HCl [pH 7.5], 10 mmol/L CaCl\(_2\), 0.02% Na\(_2\)N). Enzymatic bands were visualized after staining for 1 hour with amido black 0.1% and distained for 30 minutes with a solution of 30% (v/v) methanol and 10% (v/v) glacial acetic acid. To measure gelatinase activities, gels were read using Gel Logic 440 Imaging System (Kodak) and the density of the bands (arbitrary units) was normalized to human gelatinase control (pro-MMP-9 band) to allow comparisons between gels.

**Immunohistochemistry**

Brain samples were fixed 48 hours with formalin 10%, embedded in paraffin, and cut into 8-\(\mu\)m sections using a microtome. Paraffin was removed, endogenous peroxidases were blocked with methanol-H\(_2\)O\(_2\), and unspecific binding sites were blocked with 10% normal goat serum for 2 hours. Sections were incubated with a mouse antihuman MMP-9 1:50 (Chemicon) and a mouse antihuman collagen IV 1:10 (Masterdi-
agnostica) or mouse antihuman myeloperoxidase, myeloperoxidase, (Masterdiagnostica) for 1 hour at room temperature. Secondary antibody (goat antihorse herpaderisidase, 1:500) was applied during 30 minutes. Immunoreactive sites were developed with DAB solution and sections counterstained with Mayer hematoxylin.

Number of MMP-9\textsuperscript{+} neutrophils, number of microvessels with peripheral MMP-9\textsuperscript{+} neutrophils, and number of microvessels presenting erythrocyte extravasation (named disrupted microvessels) were counted in 20 random fields at \(10\times\) magnification per area and subject.

**Statistical Analyses**

SPSS 12.0 package was used for statistical analyses. Statistical significance was assessed by ANOVA followed by Bonferroni tests for intergroup comparisons. The Spearman coefficient was used to study correlations between continuous variables. Values are given in mean±SEM. \(P<0.05\) was considered statistically significant.

**Results**

**High Content of MMP-9 in the Hemorrhagic Infarcted Brain**

Our zymography data shows that whole brain homogenates displayed a wide variability in total MMP-9 levels (HT, 2.27±0.21; I, 1.82±0.22; and C, 0.71±0.04 band density) but a narrow difference for MMP-2 (HT, 0.44±0.08; I, 0.44±0.05; and C, 0.29±0.04 band density) was found, as Figure 1A represents. Because two clear bands could be detected for MMP-9 corresponding to pro-MMP-9 at 95kDa and a cleaved form at 85kDa, further analysis of these 2 proteins was performed (HT, 1.60±0.16; I, 1.34±0.18; and C, 0.43±0.02 for pro-MMP-9 band density and HT, 0.67±0.07; I, 0.48±0.04; and C, 0.27±0.02 for cleaved MMP-9 band density).

As Figure 1B shows, total MMP-9 levels (including MMP-9 form at 95kDa and cleaved form at 85kDa) peaked in areas of hemorrhagic transformation that contained significantly higher levels than in the contralateral hemisphere \((P<0.0001)\). Similarly, infarct areas also demonstrated elevated MMP-9 levels when compared with contralateral tissue \((P=0.001)\). No statistically significant difference was reached when comparing hemorrhagic versus nonhemorrhagic areas within the ischemic territory. However, a detailed analysis of the content of proband cleaved MMP-9 forms showed significantly higher levels of cleaved MMP-9 in the hemorrhagic areas compared to nonhemorrhagic and contralateral tissue \((P=0.033\) and \(P<0.0001\), respectively) and in nonhemorrhagic infarcts compared to contralaterals \((P=0.012;\) Figure 1C,D). No significant differences were found for MMP-2 content.
Endothelial MMP-9 Content
In microdissected microvessels, only MMP-9 was observed in the zymograms and no MMP-2 could be detected. Again, our results showed higher MMP-9 levels in the microvessels within areas of hemorrhagic transformation compared to the contralateral hemisphere \( (P < 0.012) \), but no statistically significant differences were detectable between hemorrhagic and nonhemorrhagic areas within the infarct itself \( (P = 0.51) \). Overall, MMP-9 content in infarcted areas was increased when compared to the contralateral hemisphere \( (P = 0.043) \), as Figure 2 shows.

MMP-9\(^+\) Neutrophil Infiltration Is Associated With Basal Lamina Leakage
Histological studies and immunostaining performed to localize MMP-9 (Figure 3A) showed that an important source of MMP-9 were perivascular infiltrated neutrophils, as MPO immunostaining demonstrates (Figure 3B).

The number of MMP-9\(^+\) neutrophils was significantly higher in the hemorrhagic transformation and infarct areas when compared to the contralateral site \( (P < 0.0001) \), peaking in those hemorrhagic areas when compared to the infarcts \( (P < 0.0001) \). The number of capillaries that presented neutrophil infiltration and the number of disrupted microvessels were increased in infarcted areas compared with contralateral \( (P < 0.0001) \), with the highest counts occurring in the hemorrhagic transformation areas \( (P < 0.0001) \); Figure 3C).

A regression analysis showed that total MMP-9 levels detected in whole brain by zymography were positively correlated with the number of MMP-9\(^+\) neutrophils \( (r = 0.808, P = 0.001) \), the number of microvessels presenting MMP-9\(^+\) neutrophils \( (r = 0.632, P = 0.028) \), and microvessels associated with erythrocyte extravasation \( (r = 0.673, P = 0.016) \).

To assess the involvement of basal lamina integrity, we stained the brain samples for type IV collagen. Our results
showed a strong immunoreaction in the contralateral vessels, whereas a weaker signal was observed in the infarct and hemorrhagic areas. Moreover, as Figure 4A demonstrates, we found a weaker collagen IV signal in vessels of both ischemic areas clearly associated with the presence of perivascular neutrophils in relation with collagen IV degradation. In fact, some capillaries demonstrated a complete disappearance of collagen IV immunoreaction associated with massive neutrophil MMP-9\(^+\) infiltration together with the appearance of blood erythrocytes in the parenchyma (Figure 4A). Finally, MMP-9\(^+\) neutrophils were colocalized with type IV collagen degradation in infarct and hemorrhagic microvessels, as Figure 4B shows.

**Discussion**

The present investigation demonstrates that in hemorrhagic complications that occur after human ischemic stroke, the
infiltration to the ischemic tissue by peripheral blood neutrophils filled of MMP-9 is related to the basal lamina degradation compromising BBB integrity that precedes massive blood extravasation.

HT are the most threatening complications that follow ischemic stroke, impairing the prognosis and increasing the mortality rates. At the present time, the best treatment for stroke patients is the intravenous administration of recombinant tissue plasminogen activator for the lyses of the clot at brain vessels. Nevertheless, side effects on the brain parenchyma such as hemorrhagic complications might also appear after tissue plasminogen activator administration, limiting the benefits of the thrombolytic therapy. An understanding of the mechanisms responsible for both spontaneous and thrombolysis-associated hemorrhagic events could improve actual stroke therapy but, unfortunately, this phenomenon remains to be fully elucidated. Previously, several investigations have demonstrated the partial importance of free radical generation and oxidative stress that occurs after reperfusion and damages the blood vessels wall. However, in the past years the additional role of proteases has been emphasized as another mechanism causing cerebrovascular damage, such as the MMP family.

The objective of the present study was to determine the possible contribution of MMP-9 to BBB breakdown and further hemorrhagic transformation events that sometimes occur after ischemic stroke and to identify its source in the human brain. Our results support the hypothesis of a direct role for MMP-9 in hemorrhagic transformation events because: (1) zymography shows increased levels of MMP-9 in the infarct and hemorrhagic transformation areas; (2) the content of cleaved MMP-9 (known as an active form) was particularly high in hemorrhagic areas; (3) LCM clearly demonstrates that the microvascular endothelium that form the BBB contain high levels of MMP-9 in the mentioned ischemic areas; (4) perivascular neutrophils are an important source of MMP-9 and (5) a severe degradation of a basic BBB component such as collagen IV occurs in those vessels presenting an important infiltration of MMP-9 neutrophils and erythrocyte extravasation.

The present findings confirm that increased MMP-9 is located in the vascular/perivascular vicinity of infarct and hemorrhagic transformation areas. These findings are sustained by other studies that previously showed a primary MMP-9 increase in infarct areas both in animal models and human ischemic brain. More novel are those studies using LCM techniques. An expression analysis from blood brain vessels in a middle cerebral artery occlusion model was recently performed in rats showing MMP-2 and MMP-9 mRNA upregulation in endothelial cells from the ischemic core and boundary regions. To our knowledge, the present study is the first using LCM in human brain tissue in a cerebrovascular disease, confirming the utility of the LCM. Microdissection confirms that content of MMP-9 was similarly high in endothelium from hemorrhagic and infarcted microvessels compared to contralateral hemisphere, pointing to neutrophils surrounding dissected microvessels as a significant source of MMP-9 in hemorrhagic areas.

Classically, animal studies have mainly focused their efforts in assessing MMP-9 expression in the brain after ischemia, but several studies in humans have demonstrated that this MMP-9 increase after ischemia also occurs at blood level closely related with cerebral parameters such as infarct size or cerebrovascular events as the appearance of hemorrhagic complications after tissue plasminogen activator administration. Whether the main source of this protease is resident brain cells or circulating blood cells remains unknown. Whereas few studies could not identify neutrophils as a primary source of MMP-9 protein in animal models of cerebral ischemia, other studies strongly suggest and demonstrate the implication of recruited leukocytes as a significant source of MMP-9 causing basal lamina degradation and BBB breakdown after transient ischemia in rodents. These studies have demonstrated in mouse and rat models the neutrophil infiltration to the ischemic brain contributing to the tissue inflammatory response by releasing MMP-9 and causing BBB breakdown secondary to basal lamina degradation. In fact, chimeric mice lacking leukocytic MMP-9 present similar lesions than MMP-9 knockouts but not those chimeric animals lacking MMP-9 in brain cells.

Our results showing that infiltrated blood neutrophils are an important source of MMP-9 in the ischemic areas are supported by these previous investigations. Besides, it is important to highlight that our immunostaining study shows that, in the infarct areas, there is significant vascular MMP-9 neutrophil concentration but just few disrupted vessels, which suggests that the infiltration occurs previously to the BBB leakage.

Our group has recently reported a MMP-9 neutrophil infiltration in the infarct areas after human stroke, but this is the first time that this MMP-9 is described to be associated to collagen IV degradation in human ischemic brain after hemorrhagic transformation episodes. The loss of basal lamina integrity has been postulated to be the primary cause of hemorrhage after ischemic event because MMP-9 can degrade the main components of the basal lamina such as laminin, fibronectin, and collagen IV. Other authors have demonstrated the implication of this protease in hemorrhagic events and basal lamina destruction in rat models of cerebral ischemia. Furthermore, those studies have also shown a partial protection of hypothermic treatments through the inhibition of different proteases including MMP-9 and that tissue plasminogen activator-induced MMP-9 activation is related to the destruction of basal lamina components, suggesting that its early inhibition could reduce hemorrhagic transformation events. Our results also demonstrate the association between MMP-9 and collagen IV degradation in the human brain related to hemorrhagic events.

Finally, an issue that requires further analysis is the mechanistic relationship between MMP-9 and neutrophils in our hemorrhage hypothesis. Although our results clearly show significant elevations in MMP-9–positive neutrophils in hemorrhagic versus nonhemorrhagic areas in ischemic brain territory, clearly supported by the upregulation of cleaved MMP-9 in hemorrhagic areas, is known that other brain cell types, such as neurons, glial, or endothelial cells, are partially contributing to MMP-9. Furthermore, the precise timing of neutrophil accumulation, MMP-9 secretion, basal lamina degradation, and subsequent hemorrhagic conversion is difficult to quantify. However, we demonstrate a good correlation between MMP-9 content, neutrophil infiltration, and microvessel disruption.

Despite the new findings presented in this study, it presents few limitations that should be discussed. Perhaps the main
caveat is the small number of cases, although we want to emphasize the importance of studying brain samples from strokes involving hemorrhagic events and the lack of other investigations with this kind of samples. Also, the fact that we could not perfuse the cerebral vessels with a saline solution to eliminate peripheral blood could be confusing because some MMPs are known to circulate in the blood stream. However, we believe that this contribution might be small because MMP-2 remains constitutively expressed.

Summary

Our investigation reveals for the first time the destruction of a structural component of the BBB associated to the infiltration of MMP-9 neutrophils in the ischemic brain after human stroke. Then, MMP-9 is confirmed to be a key protease interfering with BBB leakage and natural evolution of cerebral ischemia. We believe that modulate MMP-9 or neutrophil infiltration processes could be a target for future investigations to improve the present thrombolytic therapy for ischemic stroke. It would be particularly helpful in patients with salvageable tissue where exists an increased risk of hemorrhagic transformations that limits the time window for the treatment within the first 3 hours after stroke onset.

Acknowledgments

We are grateful to Manolo Quintana for statistical advice, to Maria Angeles Artaza for her always kind assistance in laser capture microdissection, and to all neurologists and residents from the Neurovascular Unit and technical staff from the Neuropathology Department who helped in performing this study.

Sources of Funding

Anna Rosell is the recipient of a postdoctoral grant from Ministerio de Educacion y Ciencia (EXT2006/766) and Eloy Cuadrado is supported by a grant from the Fondo de Investigaciones Sanitarias de Educacion y Ciencia (EXT2006/766) and Eloy Cuadrado is Anna Rosell is the recipient of a postdoctoral grant from Ministerio.

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*Stroke*. 2008;39:1121-1126; originally published online March 6, 2008; doi: 10.1161/STROKEAHA.107.500868

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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