Profiles of Matrix Metalloproteinases, Their Inhibitors, and Laminin in Stroke Patients

Influence of Different Therapies

Solveig Horstmann, MD; Pamela Kalb, MD; James Koziol, PhD; Humphrey Gardner, MD; Simone Wagner, MD

Background and Purpose—The goal of this study was to determine the temporal profile of several matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and laminin (an MMP substrate) in human stroke under different treatment paradigms, including thrombolysis and hypothermia.

Methods—We serially measured the serum levels of MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1, TIMP-2, and laminin in 50 patients with acute ischemic stroke using zymography or enzyme-linked immunosorbent assay. Patients were treated with heparin, therapeutic thrombolysis, or hypothermia. Scandinavian Stroke Scale scores were obtained at baseline. Infarct volume was measured with CT scanning on day 4 after stroke onset. Healthy persons were used as control subjects.

Results—MMP-2 and MMP-9 increased during the course of ischemia, whereas intact laminin and TIMP-2 decreased significantly (P<0.05). MMP-9 and laminin levels varied significantly by infarct size (P=0.001) and therapy (P=0.0005). MMP-9 levels were significantly higher in patients treated with tissue plasminogen activator (tPA) compared with patients treated with hypothermia. The cleaved form of MMP-9 was found solely in 4 patients treated with tPA. Intact laminin levels were significantly lower in the tPA group than in the hypothermia group.

Conclusions—Selected MMPs and TIMPs are involved in the pathophysiology of acute stroke. This is also reflected by changes in laminin. Treatment paradigms differentially influence levels of MMP-9 and laminin. Combination therapies explicitly involving MMP inhibition could be of value in future treatment strategies. (Stroke. 2003;34:2165-2172.)

Key Words: hypothermia ■ metalloproteinases ■ stroke, ischemic ■ thrombolytic therapy


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In this study, we determined the temporal profile of several MMPs and TIMPs in human stroke under different treatment paradigms, including thrombolysis and hypothermia, in relation to stroke size and severity.

Subjects and Methods

Serum MMP, laminin, and TIMP levels were measured sequentially in 50 patients with acute embolic supratentorial ischemic stroke admitted to the Department of Neurology, University of Heidelberg (Heidelberg, Germany), within the first 12 hours of stroke symptoms. Stroke patients were treated with heparin (n=18), hypothermia (n=15), or thrombolysis (n=17). Heparin was administered intravenously and adjusted to an 1.5- to 2-fold increase in partial thromboplastin time. tPA was administered at a dosage of 0.9 mg/kg IV (10% bolus, 90% infusion during 1 hour). Hypothermia was performed as described elsewhere. The decision for or against a specific therapy was made independently of our study. Patients who died before sampling was completed are not included in this study. These were 2 deaths in the tPA group, 3 in the hypothermia group, and 4 in the group treated with heparin.
Risk factors, including history of hypertension, hyperlipidemia, and smoking, were determined. To identify the potential pathological mechanism of the ischemic event, each patient underwent CT, ECG, and duplex sonography on the day of admission. Excluded from the study were patients with (1) previous history of transient ischemic attack or stroke; (2) history of recent head trauma; (3) major cardiac, renal, hepatic, or malignant diseases; (4) atherosclerotic plaques in the carotid artery; (5) obvious signs of infection after admission; or (6) diabetes mellitus. Peripheral blood samples were taken by venipuncture on admission and at days 1, 2, 4, 8, and 12 after admission. Samples were centrifuged after clotting (1500 \( \times \) g/15 min) and the serum was stored at \( -80^\circ \text{C} \) until analysis. All assays were performed with first-time thawed aliquots within 2 weeks of sampling and analyzed blindly. Fifty age- and sex-matched healthy persons, who had no recent infection, trauma, or serious illness, were used as control subjects. All routine blood parameters, including white blood cell count, fibrinogen, and glucose levels, were determined at the time of sampling. The clinical examination was scored according to the Scandinavian Stroke Scale (SSS) at admission. All our patients had medium to large infarctions of the middle cerebral artery and most had to be treated in the neurological intensive care unit (NICU). Sixty percent of our patients had to be ventilated or had sedative drugs on the first days of treatment, which did not allow follow-up scoring during this period. All patients were evaluated by CT on day of admission with a follow-up CT on day 4. We used the second CT to establish the exact size of the infarcted area. For image analysis, tapes of the CTs were analyzed with the computer program Voxel Q (Marconi Medical Systems).

### Enzyme-Linked Immunosorbent Assays

MMP-3 (Amersham Europe; detection limit, 2.35 ng/mL), MMP-13 (Amersham Europe; detection limit, 0.032 ng/mL), TIMP-1 (Amersham Europe; detection limit, 1.25 ng/mL), TIMP-2 (Amersham Europe; detection limit, 3 ng/mL), and laminin (Takara Japan; detection limit, 5 ng/mL) were measured by standard quantitative sandwich enzyme-linked immunosorbent assay (ELISA). All ELISAs were performed according to manufacturer’s recommendations. Laminin breakdown products were Western blot with the polyclonal antibody 0688b (kind gift of Professor V. Quaranta, TSRI). Western blotting was performed as described previously.\(^{14}\)

### Zymography

Gelatin and casein zymograms were performed as previously described.\(^{15,16}\) MMP-2 and MMP-9 were quantified by gelatin zymography. MMP-3 was quantified by casein zymography. The specificity of the band on casein zymography was confirmed by Western blots (MMP-3: MoAb mouse anti-human, clone 55-2A4, Oncogene), MMP-7 and MMP-10, which if present appear on casein zymography, could not be detected. Western blotting was performed as described previously.\(^{14}\)

In brief, samples were diluted 1:15, and the protein content of each sample was measured with a Bradford Kit (Bio-Rad). Sample volume was corrected for protein concentration. After electrophoresis, the zone of enzyme activity was quantified with NIH Image 1.6. The study was approved by the ethics committee of the Medical School, University of Heidelberg, and all patients or their relatives gave informed consent.

### Statistical Analysis

Summary statistics are presented as mean±SD or medians with ranges for continuous data and as absolute numbers and percentages for discrete data. One-way analysis of variance (ANOVA) was used to compare infarct sizes among the therapy subgroups. Repeated-measures ANOVA was used to examine MMP, laminin, and TIMP expression over time in stroke patients relative to control subjects and in the therapy subgroups. In this regard, square root transformations of TIMP levels were taken before analyses to induce approximate normality. Repeated-measures analyses of covariance was used to examine whether age, sex, and infarct volume were related to MMP, laminin, or TIMP expression in the 3 therapy subgroups.

### Results

#### Patients

Fifty patients with hemispheric embolic infarction (33 men, 17 women; median age, 60 years; range, 45 to 73 years) and 50 control subjects (33 men, 17 women; median age, 59 years; range, 43 to 75 years) were studied. Patient characteristics, including risk factors and biochemical and hematological data taken at the time of first sampling, are shown in Table 1.

The median initial SSS score was 18 (range, 7 to 28). The initial score did not differ significantly in the different therapeutic subgroups. CT revealed medium to large infarctions of the middle cerebral artery in all patients, with a median infarct size of 69 cm\(^3\) (range, 20 to 100 cm\(^3\)). Infarct sizes did not differ significantly among the therapeutic subgroups (F\(_{2,47}=1.82, P=0.17\)). Median infarct sizes were

<table>
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<th>TABLE 1. Characteristics of Patient Subgroups</th>
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<tr>
<td><strong>Control Subjects</strong></td>
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<td><strong>Male, n (%)</strong></td>
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<td><strong>Age (median/range), y</strong></td>
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<td><strong>Leucocytes per 1 mm(^3) (median/range), n</strong></td>
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<td><strong>Platelets ( \times 10^9 ) (median/range), n</strong></td>
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<td><strong>Glucose (median/range), mg/dL</strong></td>
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<td><strong>Infarct volume (median/range), cm(^3)</strong></td>
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<td><strong>SSS score (median/range)</strong></td>
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\(^{14}\)
65, 60, and 76 cm$^3$ in the heparin, thrombolysis, and hypothermia groups, respectively.

Leukocytes, fibrinogen, glucose, and platelet counts were not significantly different in stroke patients compared with control subjects. More stroke patients than control subjects had a history of hypertension and hyperlipidemia, although these differences were not statistically significant.

**Serum Levels of MMP-2, MMP-9, MMP-3, Their Inhibitor TIMP-2, and Their Substrate Laminin Are Altered by Cerebral Ischemia**

Levels of MMP-2, MMP-3, MMP-9, TIMP-2, and laminin were significantly altered over the time course of the study. Table 2 presents summary statistics relating to these levels. We normalized levels relative to the respective mean control values and display the normalized levels over time in Figure 1. The various patterns are quite distinctive. MMP-9 levels increase dramatically by day 1 and remain elevated until day 12. MMP-3 levels increase at day 1 and then gradually decline to control levels. MMP-2 and laminin levels evince a similar pattern, the mirror image of that of MMP-3: levels are decreased relative to control subjects at day 1 and then gradually recover to control levels. TIMP-2 levels dramatically decrease at day 2 and remain depressed through day 12. Figure 2a shows a representative zymogram displaying the time course of MMP-2 and MMP-9.

**Levels of MMP-13 and TIMP-1 Are Not Altered in Cerebral Ischemia**

MMP-13 and TIMP-1 measured by ELISA were similar in control subjects and the cerebral ischemia patients at all days of measurements without significant changes over time. MMP-13 levels were 0.56 ng/mL (SD, 0.3 ng/mL) in control subjects and 0.50 ng/mL (SD, 0.41 ng/mL) on day 1 after cerebral ischemia. TIMP-1 levels were 43.05 ng/mL (SD, 19.05 ng/mL) in control subjects and 45.00 ng/mL (SD, 23.09 ng/mL) on day 1 in stroke patients.

**Effect of Different Treatment Strategies on MMP Expression**

The 50 stroke patients could be classified into 3 subgroups, depending on treatment strategy: intravenous heparin (n = 18), moderate hypothermia (n = 15), or systemic thrombolysis (n = 17). We used repeated-measures analyses of covariance.
to determine whether MMP, TIMP, or laminin levels varied significantly between subgroups, along with the potential covariates age, sex, and infarct size. There were 2 significant findings: both MMP-9 and laminin levels varied significantly by therapy (MMP-9: $F_{2,46} = 4.76$, $P = 0.013$; laminin: $F_{2,46} = 9.89$, $P = 0.0005$) and by infarct size (MMP-9: $F_{1,46} = 4.23$, $P = 0.045$; laminin: $F_{1,46} = 12.25$, $P = 0.001$). In Figure 3 we present plots of MMP-9 and laminin levels over time separately by therapy subgroup and infarct size. MMP-9 levels tend to be highest in the thrombolysis subgroup, intermediate in the heparin subgroup, and lowest in the hypothermia subgroup. Inversely, laminin levels were lowest in the thrombolysis, intermediate in the heparin, and highest in the hypothermia subgroups. Patterns relative to infarct volumes are less clear. Generally, infarct volumes are directly related to MMP-9 levels and inversely related to laminin levels, although there are exceptions at many individual time points. The cleaved form of MMP-9 (82 kDa) was found solely in 4 stroke patients treated with tPA (Fig 2b). None of the zymograms of the patients in the other treatment groups and control subjects revealed the active forms of MMP.

**Discussion**

This study presents the first systematic endeavor to characterize the time course of MMPs, their substrate laminin, and their inhibitors TIMPs in acute stroke within the context of different treatment modalities. Our findings are complementary to previous reports showing changes in the level of MMP-9 in the peripheral blood of stroke patients, with possible implication for hemorrhagic transformation. We focused on the MMPs because they have been shown to play an important role in vascular and cardiac disease. MMP-2, MMP-9, MMP-3, laminin, and TIMP-2 show characteristic temporal profiles. In addition, MMP-9 and laminin are altered by different treatment modalities. We emphasize that our patient group is well defined with acute stroke symptoms, medium to large hemispheric infarctions, and exclusion of existing carotid disease, heart disease, and diabetes mellitus. We applied these strict criteria to examine the influence of ischemic stroke on MMP, laminin, and TIMP levels independently from risk factors such as atherosclerosis and diabetes mellitus, known to influence MMP levels. The exclusion of
patients who died before sampling was completed may introduce some bias into our findings, although the relatively small numbers of dropouts should not measurably influence the overall trends we report.

We found distinctive time courses for different MMPs, TIMPs, and laminin that were influenced by thrombolytic and hypothermic therapy.

MMP-9 showed a significant increase over the course of stroke in our patient group, and MMP-3 (stromelysin 1), a type IV collagenase also able to cleave laminin, increased significantly on day 1. Concomitant with the increase in MMP-9, an increase in laminin breakdown products and a decrease in intact laminin molecules were observed. MMP-9 and laminin levels varied by infarct size.

MMP-13 (collagenase 3) and TIMP-1 showed no detectable changes, whereas MMP-2 and TIMP-2 both showed a significant decrease at day 1 with subsequent recovery of MMP-2 to levels seen at admission.

Different pathophysiological mechanisms may explain the different profiles of the different MMPs and their inhibitors, leading to alterations in synthesis or changes in the equilibrium between serum and tissue concentrations. At the transcriptional level, most cytokines known to be involved in stroke pathophysiology may downregulate and upregulate MMPs individually.25,26

It is likely therefore that differences in cell source or in tissue distribution account for the inverse changes in MMP-2 and MMP-9, whose production may both be increased at the level of transcription. In previous animal experiments, increased amounts of MMP-2 and MMP-9 could be found in infarcted tissue.27,28 Mobilization of MMP-2, likely to be produced largely by endothelial cells, to the infarcted tissue might therefore explain the initial reduction in blood levels.29,30 Because TIMP-2 is required for activation and inhibition of MMP-2 and is expressed both coordinately with and complexed to MMP-2, TIMP-2 levels would be expected to follow MMP-2 levels in most circumstances.

On the other hand, MMP-9 and MMP-3 are likely to be produced in significant amounts by activated intravascular monocytes,31 and even if these molecules also move into the infarct, their levels are likely also to immediately increase in the blood. The lack of a parallel increase in TIMP-1 with MMP-9 in stroke could reflect the inability of the assay to detect subtle changes in TIMP-1.

With regard to laminin, it seems reasonable that upregulated MMPs would cause cleavage of basement membrane laminin and an increase in cleavage fragments seen in the blood. The source of intact laminin in the blood is less clear, but its presence suggests that some small portion of endothelial laminin production may be washed from the luminal surface of the endothelium into the bloodstream rather than being deposited in the abluminal basement membrane.

Characteristic MMP and TIMP expression patterns have been reported in a variety of other diseases. In myocardial infarction, TIMP-1 and MMP-1 show reduced expression in the peripheral blood of patients,32 whereas in the chronic failing heart, MMP-13 and membrane-bound MMP-1 (MT1-MMP) were upregulated.17 MMP-13 in this context may be derived from the large mass of activated myocardial fibroblasts, and the absence of these cells in stroke probably accounts for the lack of changes in this collagenase.

The different treatment modalities significantly affected MMP-9 and laminin levels. MMP-9 was elevated in patients treated with thrombolysis but was decreased in patients receiving therapeutic hypothermia. The increase in total MMP-9 might be an effect of tPA-mediated adherence and degranulation of neutrophils.33 The levels of laminin breakdown products paralleled this finding. Serine proteases such as plasmin can convert pro-MMPs to active enzyme.11,34 Indeed, we found the clipped form of MMP-9 exclusively in a subgroup of patients treated with tPA. Our results are complementary to findings by Montaner et al,12 who showed that baseline MMP-9 levels predict the appearance of parenchymal hemorrhage after thrombolysis. The pattern of increased expression of the latent forms of MMPs parallels previous findings in animal stroke models.18,27,28 A neuropathological study on human brain tissue,6 and the pattern of MMP expression in the cerebrospinal fluid in multiple sclerosis.35 In these reports, active forms were not detectable at all or similar to our study only in a few individuals. One reason might be that the latent forms, once released, are activated and that the activated (cleaved) MMPs are rapidly degraded. So, we might see cleaved MMP-9 only when the system is flooded.

Because MMPs are temperature sensitive,36,37 the decreased activity of MMP-9 with subsequent decreased degradation of laminin might be due to the reduced core temperature under therapeutic hypothermia. In this regard, one should note that therapeutic thrombolysis performed according to published trials excludes patients with an infarct extension of more than two thirds of the middle cerebral artery territory. Therefore, the infarcts in patients treated with thrombolysis are smaller than in those treated with hypothermia or heparin. The observed difference in our group is of importance because it is detectable even though MMP-9 varies by infarct size.

With regard to our methodology, we used zymography because zymograms are more sensitive than ELISAs and reveal active and inactive forms of MMPs. One limitation of our study is the lack of follow-up clinical scores subsequent to the score taken on admission. Many of our patients were assigned to the NICU with subsequent medication and intubation, which precluded follow-up clinical scoring. In addition, edema and increased intracranial pressure might well have contributed to observed differences after the initial event, but this is not quantifiable. Furthermore, the study is limited by the lack of pretreatment values. The first blood sample was obtained on arrival at the NICU or stroke unit. Because the window for thrombolysis is 3 hours after stroke onset, therapy is initiated immediately after CT scan. In most patients treated with thrombolysis, therapy had already been initiated by the time of blood sampling. Heparin also had been initiated before blood sampling in most patients. Baseline sampling at day 1 was before treatment only in the group treated with hypothermia.

In summary, we find characteristic changes in serum levels of selected MMPs and TIMPs that are influenced by specific treatment paradigms such as systemic thrombolysis and
moderate hypothermia. One pathophysiologic mechanism of therapeutic hypothermia may be inhibition of MMPs. Although by no means offering concrete proof, this study suggests the possibility that MMP-9 activation may be an unwanted side effect of thrombolytic therapy. This merits further exploration and might justify the use of MMP inhibitors in combination with thrombolysis.

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References
Editorial Comment

Cooling Matrix Metalloproteinases to Improve Thrombolysis in Acute Ischemic Stroke

Although their history is only recent, the role of matrix metalloproteinases (MMPs) in ischemic stroke first began to be understood when MMP expression was found to be significantly increased and implicated in blood brain barrier (BBB) disruption, edema formation, and hemorrhagic transformation in animal models of cerebral ischemia. Further study demonstrated the pharmacological blocking of these enzymes and the development of an MMP-9 knockout model, showing reduced infarct volumes and confirming its deleterious role. Recently, the participation of MMP-9 has also been demonstrated in vivo after human stroke and shown to be related to neurological worsening, infarct size, and hemorrhagic transformation.

Two innovative points are raised by Horstmann et al: first, the global study of several MMPs, together with their inhibitors and substrates, making their deleterious role more comprehensive; and second, the modification of their temporal profiles regarding different acute stroke treatments.

Concomitant with the increase in MMP-9, Horstmann et al report an increase in laminin breakdown products. This points to proteolytic degradation of critical BBB components by MMP-9, possibly explaining its deleterious role through dissolution of the basal lamina (BL). The BL matrix is constructed from type IV collagen chains and a second polymer network derived from laminin. Entactin connects both complexes, and fibronectin connects the BL with the surrounding tissue and the extracellular matrix. Parallel losses of these BL components have been shown to contribute to loss of brain microvascular integrity and are now demonstrated in human stroke.

Regarding natural MMP inhibitors, a more decisive role was expected for some of these members, such as TIMP-1, in the MMP network. However, the present study fails to show that TIMP-1 blocks the response of MMP-9 to ischemia, although the level of these inhibitors during the very first hours of stroke onset was not properly explored, and therefore its definitive contribution remains unknown.

Highlights of this study include the possibility that hypothermia modulates neuroinflammation by decreasing MMP-9 plasmatic level and weakly suggests that tPA may activate MMP-9 as an unwanted side effect. The main caveat is that treatments were initiated before blood samples were obtained. This lack of pretreatment baseline values makes it difficult to measure the effect of each intervention.

In support of this “cool” finding, there is experimental evidence that moderate hypothermia suppresses the inflammatory response. The neuroprotective effect of hypothermia is related to nuclear factor-κB inhibition, reduction of endothelial adhesion molecule expression, and leukocyte infiltration. Interestingly, the MMP-9 promoter region contains a nuclear factor-κB site, by which inflammation may regulate MMP-9 transcription. It is also known that neutrophils utilize this MMP for migration. Both facts are clear links to explain MMP-9 reduction after therapeutic hypothermia.

Another intriguing point is MMP-9 activation by tPA, suggested because cleaved forms of MMP-9 are present only in patients of the thrombolysis group. As plasmin is involved in the cascade that processes proMMP-9 to the active form, the administration of tPA may activate and promote the destructive potential of this enzyme. This hypothesis explains the reduction of tPA-induced hemorrhages after administering a MMP inhibitor (BB-94) in a rabbit model of embolic stroke. Similarly, a positive graded response exists between MMP-9 production and the degree and extent of brain bleeding after thrombolysis in human stroke.

Although some authors are researching these points extensively and generating new explanations for tPA-mediated MMP-9 activation in animal stroke models, experiments are needed to confirm the relevance of this issue in humans, with samples obtained before and promptly after tPA infusion because of the short half-life of active MMP-9 and of tPA itself.

If hypothermia decreases MMP-9 and tPA increases the expression of this molecule, a combination of both therapeutic strategies seems to be an attractive, logical approach for limiting damage from ischemia. A pilot study has already demonstrated the safety and feasibility of this combination, but a limitation in clinical practice for this combination therapy might be the reduced efficacy of tPA in the temperature range proposed for hypothermic treatment of acute ischemic stroke.

In conclusion, new studies are needed to confirm some of the interesting hypotheses proposed by Horstmann et al. Meanwhile, intensive research in the development of MMP-9 inhibitors is mandatory. At present the therapeutic time window is narrow. In the future, however, the administration of tPA to stroke patients in combination with hypothermia and MMP inhibitors even beyond 3 hours might reduce the likelihood of bleeding complications and could provide further therapeutic benefit.

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