Matrix metalloproteinase (MMP) induction and inhibition at different doses of recombinant tissue plasminogen activator following experimental stroke

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Summary

Although recombinant tissue plasminogen activator (rt-PA) is successfully used for thrombolysis in human stroke, it may increase the risk of haemorrhagic complications. It was shown that the matrix metalloproteinase (MMP) system is critically involved in basal lamina degradation after middle cerebral artery occlusion and reperfusion following rt-PA administration. We describe the effects of different doses of rt-PA (saline, 0.9, 9, or 18 mg rt-PA/kg body weight) on the MMPs, their specific inhibitors (TIMPs), and also their inducer protein EMMPRIN following experimental cerebral ischemia (3 hours [h], 24 h reperfusion, suture model) in rats. The amount of MMP-2 and -9 was measured by gelatine zymography, TIMP-1 and -2 by reverse gelatine zymography, and the content of EMMPRIN and the basal lamina component collagen type IV by Western blotting. The amount of both MMPs steadily rose with increasing doses of rt-PA (p<0.05). In contrast, their endogenous inhibitors TIMPs decreased (p<0.001). A balance between the proteases and their inhibitors was achieved at the low dose of 0.9 mg/kg rt-PA in the rats, which significantly coincided with the demonstrated protection of collagen type IV degradation at this dose. The inducer protein EMMPRIN increased in parallel to its substrate MMP-2. Exogenous rt-PA leads to an increase of the MMP-inducing system by EMMPRIN, and a rise of the degrading MMPs follows. However, at low to moderate doses of rt-PA the microvascular basal lamina was protected, probably due to inhibition of MMP-2 and MMP-9 by the upregulation of their inhibitors. This strongly supports use of the lowest effective dosage of rt-PA available.

Keywords
MMP-2/-9, TIMP-1/-2, cerebral ischemia, basal lamina, EMMPRIN

Introduction

The goal of current treatment of stroke patients with recombinant tissue plasminogen activator (rt-PA) aims to reperfuse the ischemic area. This reperfusion is caused by dissolving the fibrin of the stroke-producing thrombus and promoting blood flow. However, this beneficial effect is partially offset by a 10-fold increase in the rate of symptomatic intracerebral bleeding (1). Furthermore, delayed treatment is associated with enhanced brain injury and loss of microvascular integrity (2).

Several mechanisms have been proposed to explain the damage associated with the reperfusion of ischemic tissue. The reintroduction of oxygenated blood into the damaged region enhances the production of free radicals, recruits neutrophils and macrophages, and releases proteases (3). It has been postulated that the loss of basal lamina integrity may involve the non-cellular proteolytic systems (4). Matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) (5), play fundamental roles in the dynamic remodeling of the extracellular matrix (ECM). Proteolysis mainly depends on an imbalance between the proteases and their inhibitors. TIMPs that interfere with the activation and action of the MMPs affect the extent and duration of proteolytic damage (6). A major pathological effect of MMP-2/-9 activity in cerebral ischemia is the disruption of the blood-brain barrier (BBB) through degradation of the basal lamina (6-8). Both MMPs are upregulated in the ischemic brain and contribute to the degradation processes of the basal lamina components such as collagen IV (9-11). In an earlier work we demonstrated that MMP-2 and -9 were up-regulated in dependency with increasing rt-PA doses (9). MMPs are activated by a complex activation cascade: the proforms of soluble MMPs are activated by soluble proteases such as plasmin or other MMPs. The synthesis of some MMPs appears to be affected by the MMP-inducer protein EMMPRIN (12, 13). Re-
cently, EMMPRIN expression was detected in the ischemic brain and is potentially associated with MMP-9. It seems to be implicated in the functioning of the BBB, since EMMPRIN is preferentially located in capillaries in the brain (14).

The aim of this study was to analyze in more detail the dynamic interactions between the MMPs and TIMPs after thrombolysis with rt-PA. We combined measurements of the MMPs with those of their inhibitors, the TIMPs, after ischemia and reperfusion. Further, we assessed the influence of different rt-PA doses on the induction of EMMPRIN to test our hypothesis that these parameters might provide insights into the proteolytic potential for damaging the extracellular matrix after middle cerebral artery occlusion (MCA/O). They may also possibly explain the finding that the microvascular basal lamina is protected at low and moderate doses of rt-PA (9).

In view of the potentially wide-spread use of t-PA in stroke patients, more understanding of the balance between MMPs, their inhibitors, and their activator after cerebral ischemia may improve treatment with rt-PA.

Material and methods

All experimental procedures were approved by the government of Upper Bavaria (permission number: 211–2531/48/98) and were in accordance with animal protection guidelines. Every effort was made to reduce the number of animals used and to ensure that they were free of pain and discomfort.

Experimental groups

All experiments used male Wistar rats (250–300 g) (Charles River Laboratories, Sulzfeld, Germany), which were allowed free access to food and water. A constant period of ischemia (I) (3 hours [h]) was followed by reperfusion (R) for 24 h. A total of 24 rats were divided into four groups: the first group (n = 6) was dispensed 0.9 mg/kg body weight; the second group (n = 6), 9 mg/kg, a dose almost equivalent to the dose of 0.9 mg/kg in humans, because human rt-PA has 10-fold lower fibrin-specific enzymatic specificities in the rodent than in the human system (15); the third group (n = 6) received 18 mg/kg weight; the fourth group (n = 6) was treated with isotonic saline and served as control (I3R24). A fifth group of animals (n = 6) that had undergone sham operation served as a second control.

Preparation protocol

For details see (8). In brief, the rats subjected to R24 were intubated and anesthetized with isoflurane inhalation. Rats were not allowed to wake up and were kept under general anesthesia until reperfusion started. All animals underwent 3 h of I. Transient I and R were induced using a modified intravascular filament model according to the surgical procedure of Longa et al. (16). At the end of the reperfusion period the rats were anesthetized again and killed by transcardial perfusion. The brains were removed immediately, and the skull base was inspected for haemorrhage.

Consecutive coronal cryosections (10 µm thick) in the region 0.0 mm up to +1.0 mm distant from the bregma (stereotactic coordinates followed that of Paxinos and Watson [17]) were prepared for further analysis.

Zymography and reverse zymography

All steps for brain tissue homogenization and centrifugation were performed at 4°C. For reverse zymography, brain material gained from cryosections was homogenized in a lysis solution of 30 mM Tris, pH 8.1, and 0.5% Triton-X-100. For gelatin zymographic analysis, brain material was homogenized with 2-ml Teflon homogenizers in a lysis buffer 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij-35, 0.02% Na₂SO₄, 1% Triton X-100 (18). Protease inhibitor PMSF (100 µg/ml) was added to prevent protein degradation. Samples were then sonicated for 10 seconds and spun at 13,000 rpm for 10 minutes (min). For the non-reducing SDS-PAGE the gels (7.5% SDS–polyacrylamide gels) of the gelatin zymography contained 0.5% gelatin (Biorad). Gels for the reverse zymograms included conditioned medium (0.5 µl/ml) from HT1080 (human fibrosarcoma) cells in the gel mix together with the gelatin (19). After electrophoretic separation of brain extracts the enzymes were renatured in the gel with 2.5% Triton X-100 (1 h, room temperature), and the enzymatic digestion took place in an incubation buffer: 5 mM CaCl₂, 50 mM Tris/HCl, pH 7.4, 200 mM NaCl, and 0.2% Brij 35 for 24 h at 37°C. By staining the gels with Brilliant Blue R the MMPs were visualized (for details see [12]) as lysis zones and the TIMPs as dark bands. Reverse gelatin zymography revealed the expression of TIMP-1 and TIMP-2 in brain tissue. The reverse zymograms showed dark bands at 28 kDa and 21 kDa of TIMP-1 and TIMP-2, respectively. These zones of undigested gelatine that stained with Coomassie blue represented areas of MMP inhibition. Molecular standards (Biorad, Munich, Germany) and recombinant human MMP-2 and MMP-9 standards (Sigma, Germany) were used to calibrate molecular weights. Protein concentrations were routinely determined by a protein assay (Pierce, USA) to ensure equal protein loading to the gels.

Western blot analysis and volumetry

Brain lysates (20 µg of protein) were electrophoresed on a 10% reducing polyacrylamide gel and transferred to a PVDF membrane (Sequi-Blot; Biorad, Germany). Prior to immunoblotting a Ponceau S staining (5 min; 0.1% Ponceau in 5% acetic acid; Sigma-Aldrich) showing the overall protein loading of the blots was done. EMMPRIN was detected using a polyclonal goat anti-EMMPRIN antibody at a dilution of 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Biorad standard proteins served as control for the molecular weight.

Collagen type IV was detected using goat anti-collagen type IV antibody at a dilution of 1:500 (Southern Biotechnology, USA).

The loss of MAP-2 is coincident with neuronal loss and parallels ischemic injury (20). MAP-2 immunostaining (anti-MAP-2; Chemicon Int., USA) was used to delineate the borders of the ischemic lesion and to calculate the infarct volume (21, 22).

Analysis of the results

The different bands from the zymography were scanned and analyzed with an optical analysis program (TINA, Version 2.08, Raytest Isotopenmessgeräte GmbH, Germany) by optical densitometry. Results were displayed on an arbitrary optical density scale. The data were expressed as relative lysis zone (pixels) per µg protein and per mm lane width. The ratio of the data on the ischemic to the non-ischemic area was determined.
**Statistical analysis**

Data were expressed as means ± standard error of the means (SEM). For each group of animals and brain regions the ipsilateral was compared to the contralateral area by a Mann-Whitney U-test with a level of significance of 5%. Comparisons between the experimental groups were made with an ANOVA test (Kruskal-Wallis analysis). Percent values are given; 100% was the non-ischemic control region (contra-lateral) in each animal.
Results

A total of 30 rats survived the surgery; one animal died due to a technical failure. These rats with confirmed cerebral infarctions underwent further analysis. Six animals from each group (sham-operated, saline-treated control [I3R24], 0.9, 9, 18 mg/kg body weight) were assessed.

Figure 1A shows representative reverse zymograms from brain tissue from rats after I3R24 treated with different doses rt-PA, separated into ischemic and non-ischemic cortex and basal ganglia. The zones of MMP inhibition at 28 and 21 kDa were inhibited by 1,10 phenanthroline, a specific MMP inhibitor (data not shown), thus indicating that the bands represented MMP inhibitors. The amount of endogenous TIMP-1 and TIMP-2 in the ischemic cortex and basal ganglia after I3R24 was significantly increased compared to sham-operated animals (p < 0.05) (Table 1).

Gelatin zymographic studies from brain tissue of ischemic and non-ischemic basal ganglia and cortex provided clear evidence of pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) and in the ischemic tissue a lower-molecular-weight band of 68 kDa from active MMP-2 (Fig. 1B). The active MMP-2 was often very faint. It was not detected in the non-ischemic brain and only sometimes in the ischemic tissue. For quantification the integrated density of pro-MMP-2 was used as they are direct correlated (see also [23]).

In animals with I3R24, a mild but significant increase of MMP-9 was detected after I3R24 in the ischemic cortex and basal ganglia compared to the sham group (9). MMP-2 after I3R24 was increased in the cortex but no significant change in the basal ganglia was seen (Table 1).

Western analysis showed a significant increase of the MMP-inducer protein in the ischemic basal ganglia and cortical area after I3 R24 relative to the non-ischemic control side (both p<0.05) (Table 1).

Increasing amounts of exogenous rt-PA caused a decrease of both TIMPs (Fig. 1A). At the highest dose of rt-PA (18 mg/kg

| Table 1: Amount of TIMP-1/-2, MMP-2/-9, collagen type IV and EMMPRIN in rats with and without I3R24 (sham-operated). Level of TIMP-1/TIMP-2, MMP-2/-9, collagen type IV and EMMPRIN in the cortex and the basal ganglia of sham-operated animals (no ischemia) and animals with I 3 R24. The lytic zones after gelatin zymography (MMPs), the bands from the reverse zymograms (TIMPs), and the signals from the Western blots (EMMPRIN) were expressed as optical density (OD) values, and the ratio of the ischemic to the non-ischemic side was calculated. The values are mean and standard error of the mean of six independent animal experiments. The significance is added (Mann-Whitney U-Test).

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated</th>
<th>I3 R 24</th>
<th>MMP-2</th>
<th>MMP-9</th>
<th>Collagen IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>105 ± 4%</td>
<td>p = 0.048</td>
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<tr>
<td>basal ganglia</td>
<td>92 ± 10%</td>
<td>p = 0.027</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TIMP-2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>cortex</td>
<td>103 ± 6%</td>
<td>p = 0.045</td>
<td></td>
<td></td>
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<tr>
<td>basal ganglia</td>
<td>100 ± 15%</td>
<td>p = 0.003</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EMMPRIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>92 ± 13%</td>
<td>p = 0.025</td>
<td></td>
<td></td>
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<tr>
<td>basal ganglia</td>
<td>97 ± 12%</td>
<td>p = 0.014</td>
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| Table 2: Comparison of MMP-2/-9 and TIMP-1/-2 in rats with different doses of rt-PA. The relative amount of the ischemic to the non-ischemic side of of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the cortex and the basal ganglia of all groups (saline-treated controls and animals with 0.9, 9, and 18 mg rtPA) is shown. The bands after reverse gelatine zymography were expressed as optical density (OD) values, and the ratio of the ischemic to the non-ischemic side was calculated. The values are mean and standard error of the mean of six independent animal experiments. The multi-comparison analysis between the groups was done by ANOVA analysis.

<table>
<thead>
<tr>
<th></th>
<th>MMP-2</th>
<th>MMP-9</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortex</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.0003</td>
</tr>
<tr>
<td>basal ganglia</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.0003</td>
</tr>
<tr>
<td>Control</td>
<td>116 ± 5%</td>
<td>109 ± 4%</td>
<td>123 ± 3%</td>
<td>115 ± 4%</td>
</tr>
<tr>
<td>0.9 mg rt-PA</td>
<td>137 ± 1%</td>
<td>134 ± 1%</td>
<td>137 ± 9%</td>
<td>113 ± 7%</td>
</tr>
<tr>
<td>9 mg rt-PA</td>
<td>180 ± 3%</td>
<td>147 ± 7%</td>
<td>144 ± 7%</td>
<td>130 ± 6%</td>
</tr>
<tr>
<td>18 mg rt-PA</td>
<td>252 ± 2%</td>
<td>159 ± 17%</td>
<td>162 ± 6%</td>
<td>146 ± 5%</td>
</tr>
</tbody>
</table>
body weight) no difference between the ischemic and the non-ischemic brain areas was measured; the ratio of the ischemic to the non-ischemic side was about 1 (Table 2). In contrast, both gelatinases MMP-2 and -9 increased in the ischemic region with rising rt-PA (Table 2). When the total inhibitor level (mean of both TIMPs) and the two MMPs were plotted against rt-PA, the two graphs intersected at the lowest dosage of rt-PA. At this dosage the inhibitors compensate the proteolytic activity of the MMPs. This was shown for the overall inhibitor and proteolytic level of the whole hemispheres (see Fig. 2). These results remain valid, also when this ratio was calculated separately for the cortex and basal ganglia region (data not shown).

Western blot analysis for anti-collagen type IV (Fig. 1D) revealed that there was a significant loss of collagen type IV during I3R24: up to 64 ± 4% (cortex) and 43 ± 9% (basal ganglia) (p< 0.05). At the lower doses of rt-PA (0.9 mg/kg and 9 mg/kg) the loss of collagen type IV was significantly less compared with the control. The degradation of collagen type IV at the high dose of rt-PA was similar (18 mg/kg) to that in the control group. This indicated a protection of collagen type IV from degradation at the lower dose of rt-PA (Table 3 and [9]).

The achieved balance between the proteases and their inhibitors at the dose of 0.9 mg corresponded with the protection from damage observed at this dose (Fig. 2).

This balance was evaluated by calculating the ratio of the total inhibitor level versus the MMP levels of both MMP-2 and -9 (Table 4). After I3R24 ratio compared to sham-operated rats was significantly elevated (p<0.05) and >1 in both the cortex and the basal ganglia following ischemia and reperfusion: the TIMP/MMP balance shifted toward inhibition. With rising doses of rt-PA, this ratio declined toward a ratio below 1, showing that the balance changed into proteolytic action. The ratio of TIMP/MMP equaled 1 at the lowest rt-PA dosage of 0.9 mg/kg body weight (Table 4).

Western Blot analysis showed a significant increase of the MMP-inducer protein in the ischemic brain. This increase of EMMMPRIN rose significantly with exogenous rt-PA (Table 3) and coincided with the demonstrated increase of MMP-2 and MMP-9.

**Discussion**

In this study we examined the effect of different doses of recombinant t-PA on the MMP induction and inhibition systems after transient MCAO in rats. Three major findings were obtained. First, the amounts of MMP-9/MMP-2 as well as their endogenous inhibitors TIMP-1/-2 were increased after transient ischemia and reperfusion. Second, as the rt-PA dose increased, the MMPs rose and TIMP-1 and TIMP-2 decreased. A balance was achieved between both MMP-2/-9 and their inhibitors at the rt-PA dose

**Figure 2:** Optical density of the reverse zymograms, the gelatin zymograms, and the collagen type IV Western blots was measured. Plot of the overall level of both TIMPs in the whole hemisphere to the proteolytic activity of MMP-2 and -9 against exogenous rt-PA. Additionally the relative amount of collagen type IV in the brain tissue was calculated from the Western blots. The ratio between the ischemic and the non-ischemic sides were calculated for all groups (I3R24 control group and 0.9, 9, and 18 mg rt-PA). Data are expressed as mean value of n=6 experiments (± SEM). The multicomparison analysis between the groups was done by Kruskal-Wallis analysis; **p < 0.001.**

Table 3: Collagen type IV and EMMMPRIN in rats with different doses of rt-PA. Reduction of collagen type IV expression and increase of EMMMPRIN in the cortex and the basal ganglia depending on the amount of rt-PA given (saline-treated controls and animals with 0.9, 9, and 18 mg rtPA). The bands of the Western blots were expressed as optical density (OD) values, and the ratio of the ischemic to the non-ischemic side was calculated. The values are mean and standard error of mean of six independent animal experiments. The multicomparison analysis between the groups was done by ANOVA analysis.

<table>
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<tr>
<th></th>
<th>Collagen type IV</th>
<th>EMMMPRIN</th>
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<tr>
<td></td>
<td>cortex</td>
<td>basal ganglia</td>
</tr>
<tr>
<td>Control</td>
<td>64 ± 4%</td>
<td>43 ± 9%</td>
</tr>
<tr>
<td>0.9 mg rt-PA</td>
<td>89 ± 6%</td>
<td>79 ± 3%</td>
</tr>
<tr>
<td>9 mg rt-PA</td>
<td>81 ± 12%</td>
<td>72 ± 9%</td>
</tr>
<tr>
<td>18 mg rt-PA</td>
<td>59 ± 9%</td>
<td>57 ± 11%</td>
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</table>

**Table 4:** Relationship of the TIMPs to the corresponding MMPs of rats with and without I3R24 (sham-operated) and treated with different rt-PA doses (0.9, 9, 18 mg/kg). TIMP-1 (28-kDa band) and TIMP-2 (21-kDa band) were correlated to the activities of the both MMPs. The statistical analysis between the groups was done by Kruskal-Wallis analysis.

<table>
<thead>
<tr>
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<th>TIMPs/MMPs</th>
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<tr>
<td>Sham-operated</td>
<td>1.11 ± 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>1.40 ± 0.08</td>
</tr>
<tr>
<td>0.9 mg rt-PA</td>
<td>0.99 ± 0.05</td>
</tr>
<tr>
<td>9 mg rt-PA</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>18 mg rt-PA</td>
<td>0.60 ± 0.08</td>
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of 0.9 mg/bw, which coincided with the observed basal lamina protection. Third, EMMPRIN increased at the protein level and continued to rise with increasing rt-PA concentrations.

The MMP-system is intimately involved in damage to the microvasculature, which leads to haemorrhagic transformation and cerebral edema (10). MMPs attack the basal lamina in the cerebral capillary wall, which contains type IV collagen, fibronectin, laminin, and heparan sulphate. Under conditions of global ischemia, MMP-9 appears to also play a deleterious role, since both pharmacological inhibition and gene deletion of MMP-9 are neuroprotective in vivo (24). For example, MMP-9 knockout mice are protected from brain trauma, focal cerebral ischemia, and transient global cerebral ischemia (24-26). Exogenous tPA upregulates brain MMP-9 in vivo after transient focal cerebral ischemia, and ischemic MMP-9 levels are reduced in tPA knockouts (27). MMPs are regulated at several levels, e.g. transcription, translation, secretion, and activation (28). While MMP activity is inhibited non-specifically in the plasma by α2-macroglobulin (29), it is inhibited specifically in the tissue by TIMPs (6). TIMPs bind to the active site of MMPs in a 1:1 stoichiometric ratio and form non-covalent bonds with a very high Kd. The binding of TIMP to an MMP blocks the active site and prevents substrate activation (30). TIMP-1 primarily forms complexes with MMP-9, while MMP-2 binds to TIMP-2 (31). The expression of the TIMPs modulates extracellular matrix turnover. However, the exact mechanism of regulation and interaction of MMPs and TIMPs in vivo is complex and remains to be clarified. It is important to note that MMP expression is not always indicative of net proteolytic activity, since the latter is an outcome of expression levels, post-translational activation, and extracellular inhibition of the MMPs (32). In the zymograms we detected mainly the inactive pro-form of MMP-2. Activated MMP-2 was not detected in the non-ischemic brain tissue. Some faint bands in the ischemic brain at the molecular weight of 68 kDa were seen. This evidence of active MMP-2 in the ischemic tissue was observed in approximately half the cases. This corresponds to the observations of Hoe et al. (23) and Yang et al. (33). However, it is assumed that an increase in pro-MMPs reflects an increase in active MMPs in vivo (23, 34, 35). Failure to extract active forms from the ischemic tissue is well reported (36-38).

In this study we found a modest increase of both MMPs and TIMPS after cerebral ischemia and reperfusion, thus confirming the results of Rosenberg et al. (6). Furthermore, this increase is consistent with the finding that the TIMP-1 level is elevated in the plasma of stroke patients and also in different rat models (6, 39, 40). All these findings indicate that endogenous protection causes the system to attempt to inhibit the proteolysis and to reduce the MMP damage after the proteolytic outburst. In contrast to our rat model, the levels of TIMP-2 in stroke patients were not different from those of controls (40). However, it was shown recently that the expression patterns of MMPs and TIMPS appear to depend on the type of ischemic insult (focal vs. global) and the period and severity of the ischemic event (32). In our model of transient ischemia the ratio of the TIMPs to MMP-2 and -9 was higher than 1 after 13R24, thus demonstrating a more severe up-regulation of the overall inhibition by the TIMPS than of the MMPs. The increased activation of MMP-2 could also be due to a coordinated action of MT1-MMP and TIMP-2 (41). MMP-2 activity may, however, be limited by the parallel increase in TIMP-2 proteins. Although TIMP-2 is an inhibitor of MMPs, paradoxically it can function as a co-activator of proMMP-2 depending upon the concentration of TIMP-2 protein (42).

The second part of our study focused on the impact of exogenous rt-PA on TIMP-1/-2 as well as MMP-2 and -9. With increasing doses of rt-PA, both proteases increased steadily in the cortex and the basal ganglia, showing a reciprocally proportional behavior to both TIMPs. By correlating the results for the proteases to their inhibitors, we showed that a specific rt-PA dose compensated for the protease. The optimal rt-PA dose, i.e. the dose with the lowest net protease capacity in brain tissue, turned out to be the very low dose of 0.9 mg/kg body weight in rats. This corresponds to the dose of 0.09 mg/kg body weight in humans (15). At this dose the concentration of MMP-2 and MMP-9 in the ischemic area seemed to be neutralized, at least partly by TIMPs. However, this study is restricted to the two gelatinases MMP-2 and -9, neglecting the possible role of other MMPs like MMP-3 which is assumed to play an additional deleterious role during the degradation processes.

Our results agree with earlier published results from our group which showed that the damage of the basal lamina depended on different doses of rt-PA. We demonstrated that collagen type IV degradation and also microvascular damage were decreased at the dose of 0.9 mg rt-PA compared to higher doses of rt-PA (9, 43). Similar results were obtained from Yang et al. in a three-vessel-occlusion model of 90 min ischemia (44). This finding is supported by a Japanese study which found that the low dose of 0.6 mg/kg alteplase was effective in stroke patients (45).

The third aspect of our study was that endogenous rt-PA induced EMMPRIN. This protein is possibly associated with the induction of MMP-2, -3, and MT1-MMP in addition to MMP-1 (46-48). The present study highlights the role of EMMPRIN in ischemic, microvascular damage. Specifically, our study shows that EMMPRIN is quantitatively upregulated under ischemic conditions in rats. With higher doses of rt-PA, the protein amount of EMMPRIN in the ischemic tissue rises significantly. This increase could be responsible for the observed rise of MMPs after rt-PA treatment. How EMMPRIN induces expression of MMP remains unclear. The inducer might become active only in the presence of co-stimulators (48). EMMPRIN was shown not to induce MMP-9 directly, nor to induce TIMP-1 and TIMP-2 (12).

A recent study demonstrated that EMMPRIN also stimulated the serine uPA proteinase system (49). The activation of uPA signifies that there is an additional degradation pathway that enhances the induction of MMP and consequently its potential to damage the basal lamina. EMMPRIN thus appears to be a major regulator of both systems (49). Together with the results demonstrated in our study, this suggests that EMMPRIN inhibition could be a potentially useful target in therapy for stroke patients.

The action of thrombolysis in acute stroke is ambivalent. On the one hand, it helps to restore blood flow and improves the clinical outcome. On the other, it causes more edema and haemorrhage. The dosage of this highly effective, but also potentially dangerous drug may be the one major variable with which the clinician can influence its ambivalence. Understanding the degradation processes may lead to new ways to stop the devastating proteolytic processes. Our findings on microvascular
basal lamina damage caused by proteases following experimental stroke strongly support use of the lowest effective dosage available. Further studies will need to focus on the crucial time pattern of drug application and the activation system of degrading proteases.

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