Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Neurology 200 (2006) 38–49
Experimental Neurology
www.elsevier.com/locate/yexnr

Matrix metalloproteinase activation and blood–brain barrier breakdown following thrombolysis

Melissa A. Kelly [a], Ashfaq Shuaib [a,b], Kathryn G. Todd [a,b,c,*]

[a] Center for Neuroscience, University of Alberta, Edmonton, AB, Canada
[b] Department of Neurology, Faculty of Medicine, University of Alberta, Edmonton, AB, Canada
[c] Department of Psychiatry, University of Alberta, Edmonton, AB, Canada

Received 19 October 2005; revised 9 January 2006; accepted 12 January 2006
Available online 19 April 2006

Abstract

Thrombolysis with tissue plasminogen activator (tPA) is the only pharmacotherapy available for cerebral ischemia. However, the use of tPA can increase the risk of hemorrhage due to blood–brain barrier (BBB) breakdown. Recent evidence suggests that increased activation of matrix metalloproteinases (MMPs) may be involved in this breakdown. This study examines the temporal profile of MMP-2 and -9 following tPA administration to ischemic rats.

Male Sprague–Dawley rats were randomly assigned to one of four groups (Sham-tPA; Sham-Saline; Ischemia-tPA; Ischemia-Saline; group $n = 6$, total $N = 120$). Focal embolic ischemia was induced by middle cerebral artery occlusion through injection of an autologous clot. One hour post-surgery, tPA (10 mg/kg) or saline was delivered intravenously and animals were euthanized at 3, 6, 12, or 24 h after onset of ischemia. Infarct volume was measured by TTC staining; BBB components examined immunohistochemically; and MMP activation measured by gelatin zymography.

Our results show that tPA significantly reduced infarct volumes (overall infarct volume-Sham-tPA: 5.80 ± 4.55 [mean ± SE]; Sham-Saline: 5.00 ± 4.23; Ischemia-tPA: 186.1 ± 73.45; Ischemia-Saline: 284.8 ± 88.74; all $P < 0.05$). Treatment with tPA was also associated with the activation of MMP-9 at 6, 12, and 24 h following ischemia. No temporal changes were observed in MMP-2 activation, although tPA administration increased its activity compared to saline treatment. Analyses of immunohistochemistry showed that destruction of components of the BBB followed MMP-9 activation. Thus, increased MMP-9 activation may, in part, be responsible for the increases in hemorrhagic transformation reported with use of tPA. Our study is the first to demonstrate the temporal profile of MMP activation following thrombolysis with tPA in a model of thrombotic focal cerebral ischemia.
© 2006 Elsevier Inc. All rights reserved.

Keywords: Focal cerebral ischemia; tPA; MMP; BBB breakdown

Introduction

Thrombolysis with tissue plasminogen activator (tPA) is the only pharmacological treatment option currently available for the treatment of ischemic stroke. When administered intravenously within 3 h of symptom onset, tPA has been shown to be beneficial (Pfefferkorn and Rosenberg, 2003), albeit to a small population of patients. However, reperfusion with tPA has been associated with devastating sequelae including, an increased risk of hemorrhagic transformation and edema (Vivien and Buisson, 2000; Yepes et al., 2003). Though the molecular mechanisms underlying this transformation have yet to be fully elucidated, hemorrhagic conversion occurs as a result of the destruction of the blood–brain barrier (BBB) and an increase in vascular permeability (Yepes et al., 2003).

Matrix metalloproteinases (MMPs), a family of zinc-dependent proteases, have recently been implicated in the reperfusion injury that follows cerebral ischemia (Wang et al., 2003; Sumii, 2002). Activation of these MMPs is thought to be partially responsible for the breakdown of the BBB, due to their

* Corresponding author. Neurochemical Research Unit, Department of Psychiatry, University of Alberta, Edmonton, Canada AB T6G 2R7. Fax: +1 780 492 6841.
E-mail address: kgtodd@ualberta.ca (K.G. Todd).

0014-4886/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2006.01.032

ability to degrade components of the extracellular matrix (Yepes et al., 2003; Romanic and Madri, 1994; Tsuji et al., 2005). It is possible that tPA activates MMPs through the plasminogen–plasmin system, thereby weakening blood vessels and predisposing them to rupture (Pfefferkorn and Rosenberg, 2003; Sumii, 2002; Tsuji et al., 2005). Matrix metalloproteinase-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are the focus of many studies of cerebral ischemia because of their substrate specificity for fibronectin, laminin, and collagen type IV, structural components of the BBB (Heo et al., 1999). To date, no study has examined the temporal profile of MMP activation and BBB breakdown following a tPA-treated focal ischemic event.

The objective of this project was to determine the time course of activation of both MMP-2 and -9 as well as the BBB breakdown within the first 24 h following the administration of tPA in a rat model of focal embolic cerebral ischemia. The embolic stroke model we use here is well established in stroke research and more closely mimics human stroke than other models of cerebral ischemia (Krueger and Busch, 2002; Zhang et al., 1998). The model uses thrombin mixed with arterial blood drawn from the middle cerebral artery to create a reproducible and predictable thromboembolic occlusion.

Our results demonstrate an increased activation of MMP-9 in tPA-treated animals at 6, 12, and 24 h following ischemic injury. The activation of MMP-2 remained unchanged throughout all time points studied, although when compared to saline treatment, tPA administration increased MMP-2 activity. The destruction of structural proteins of the BBB, namely, laminin and fibronectin, closely followed the activation of MMP-9. Taken together, our results suggest that early inhibition of MMP-9 could reduce the amount of BBB damage and thereby the hemorrhagic transformation conferred by tPA administration.

Materials and methods

Male Sprague–Dawley rats (250–300 g) were housed in a humidity and temperature-controlled environment with a 12-h light/dark cycle. The care and use of animals were in compliance with the guidelines as outlined by the Canadian Council for Animal Care. Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad, during surgical procedures. Animals were randomly assigned to one of the following four experimental groups: Sham-tPA; Sham-Saline; Ischemia-tPA; Ischemia-Saline. Each of these four groups included all five time points of interest: 3, 6, 9, 12, and 24 h following induction of ischemia or sham operation. Total $N = 120$; group $n = 6$.

Focal cerebral ischemia

Animals were anesthetized using halothane (3% to induce and 1% to 1.5% for maintenance), 70% oxygen, and 30% nitrogen. For this study, we used the thromboembolic model of focal cerebral ischemia (Wang et al., 2001). Briefly, a surgical incision was made in the ventral neck to expose the common, internal, and external carotid arteries. The external carotid artery (ECA) was fully ligated for the insertion of a PE-10 catheter. The catheter was advanced through the internal carotid artery (ICA) to the distal opening of the middle cerebral artery (MCA). Blood was drawn from this region and mixed with thrombin contained in the catheter to form a clot. Clot formation was allowed to progress for 15 min and the clot was deposited followed by a small saline injection to advance the clot to the middle cerebral artery. The catheter was withdrawn after 15 min, animals were then returned to their cages for recovery. Those animals randomly assigned to a sham group received the same surgical procedure with the exception of clot formation and deposition. Animals were monitored behaviorally continuously throughout the recovery period and were scored using a modified Bederson's scoring system as described in Bederson et al. (1986).

One hour following the surgical procedure, animals were again anesthetized using 1% halothane and received a tail vein injection of either tPA (10 mg/kg) or saline. The administration of tPA was in conjunction with clinical guidelines, human recombinant tPA (Actilyse) is 10 times less potent at activating rodent plasminogen than it is at activating human plasminogen (Benchenane et al., 2004; Lijnen et al., 1994), therefore we use a dose 10 times more concentrated (10 mg/kg).

Three, six, twelve, or twenty-four hours following the surgery, animals were euthanized and brains quickly removed and blocked for analyses of infarct volume, breakdown of BBB components, and regional activation of MMP-2 and -9.

Measurement of infarct volume

One 2-mm coronal section of brain in the known infarct zone (Bregma +0.20 mm according to The Rat Brain Atlas (Paxinos and Watson, 1998) was incubated in 2,3,5-triphenyltetrazolium chloride (TTC) at room temperature. Stained tissue sections were scanned using HP ScanJet software and converted to jpeg file in Adobe Photoshop. The volume of infarcted tissue was quantified using a standard image analysis technique where the freehand tool was used to trace the infarcted area measured in pixel size (Krueger and Busch, 2002; Tureyen et al., 2004; Swanson et al., 1990). The possibility of edema enlarging the infarcted tissue is taken into account by measuring the area of the surviving tissue on the ipsilateral side and subtracting it from the area on the contralateral side (Swanson et al., 1990). TTC staining as a measure of infarct volume is commonly used and is equivalent to the cresyl violet or hematoxylin–eosin staining methods for quantifying infarct volume following focal cerebral ischemia (Tureyen et al., 2004; Bederson et al., 1986).

Gelatin zymography

Remaining brain tissue containing the infarct zone was immediately flash-frozen in ice-cold isopentane and stored at −80°C until used for further histological analyses. Specifically, one set of brains ($n = 3$) from each of the four groups and each of the four time points were used for gelatin zymography (total $N = 60$). Another set of brains ($n = 3$ from all groups and

time points examined; total $N = 60$) was used for cryosectioning and immunostaining. Tissue used for gelatin zymography was divided into ipsilateral ischemic hemispheres and contralateral non-ischemic hemispheres and further dissected into striatal and cortical regions. This dissection process was performed to thoroughly compare MMP activation throughout both the core infarct area (striatum) and the penumbral infarct area (cortex) over time. Brain samples were homogenized in lysis buffer containing 50 mM Tris–HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 2 μg/mL aprotinin. After centrifugation at 9000 rpm for 20 min, supernatant was collected and total protein concentration determined by Bradford's protocol. Equal amounts (40 μg/μL) of total protein extracts were prepared by mixing with lysis buffer and a loading reagent. Prepared samples (2 μg/μL) were loaded and separated by 8% acrylamide gel containing 0.1% gelatin as a substrate. Purified human MMP standards (Oncogene) were loaded in one lane at a final protein concentration of 10 μg/100 μL. Upon separation by electrophoresis, gels were incubated in renaturing buffer (Triton-X 100 2.5% v/v) for 1 h. Gels were then incubated in buffer solution (50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$) at 37°C until bands appeared. Gels were stained with Coomassie blue R-250 for 1 h and destained accordingly. MMP activation appeared as transparent bands on a blue background. Images of gels were captured by scanning each gel on an HP ScanJet flatbed scanner; bands were quantified by densitometry using ImagePro Express Analysis system.

IgG extravasation

This technique is used as a gross measure of BBB breakdown (Todd and Butterworth, 1999; Calingasan et al., 1995). By incubating slides with species-specific IgG, one can microscopically visualize and quantify the extent of leaky blood vessels as the IgG contained within vessels leaks into the parenchyma. Frozen coronal sections (20 μm) were obtained from brain tissue not incubated with TTC ($n = 3$, each time point). Sections were fixed in formalin and dehydrated through a series of graded alcohols and xylene. Sections were then incubated for 1 h at room temperature with anti-rat IgG (1:250 in PBS). Peroxidase activity was visualized with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide in PBS. Blood vessel leakiness was quantified by field scoring areas known to be in the infarcted area.

Immunohistochemistry

Immunohistochemistry was performed to assess the cellular localization of MMP-2 and -9, and to determine extent and location of the degradation of both laminin and fibronectin. Vascular integrity was measured by a loss of positive staining for these two structural components of the BBB. This was performed for all groups at all time points under study. Frozen coronal sections (20 μm) were obtained from brain tissue that was not incubated with TTC ($n = 3$, from each time point). Sections were fixed in formalin (except when antibodies recognizing MMP-2 and -9 were used) and were dehydrated through a series of graded alcohols and xylene. Quenching of endogenous peroxidase activity was achieved through incubation in 50% methanol containing 0.03% hydrogen peroxide. Non-specific binding was blocked with a universal blocking solution and 0.2% triton-X. Sections were then incubated overnight with primary antibodies, these included: anti-laminin (1:100); anti-fibronectin (1:600); anti-MMP2 (1:50); anti-MMP9 (1:100); anti-glial fibrillary acidic protein (GFAP) (1:1000); and anti-NeuN (1:250). Sections were washed with PBS and incubated with appropriate secondary antibodies (1:200). Those secondary antibodies not conjugated to fluorescent chromogens were incubated for 30 min with an avidin–biotin complex and visualized by incubation with DAB and hydrogen peroxidase. Negative controls were subject to all treatment conditions with the exception of primary antibody incubation. Loss of laminin and fibronectin was quantified by counting positive staining in striatum and cortical regions of both ipsilateral and contralateral hemispheres.

In situ zymography

This technique was used to localize net gelatinolytic activity. FITC-labeled DQ gelatin is used as a substrate for gelatinases. Cleavage of gelatin substrate by gelatinases results in increased intensity of fluorescence through release of the quenched fluorochrome. Tissue sections were obtained as described above. Thawed sections were incubated for 24 h in reaction buffer (0.05 M Tris–HCl; 0.15 M NaCl; 5 mM $CaCl_2$; 0.2 mM NaN_3; pH 7.6), containing 40 μg/mL DQ gelatin. Sections were then fixed in formalin and coverslipped with gel mount. Slides used as negative controls were incubated with reaction buffer not containing DQ gelatin. Fluorescence was visualized under fluorescence microscopy and photographs obtained with CoolSnap Pro digital camera and Image Pro Plus Software.

Statistical analyses

Quantitative data were expressed as mean + SEM. Statistical comparisons for infarct size were done using two-way ANOVA followed by Bonferroni post hoc analyses. Comparisons for laminin and fibronectin staining were performed using the non-parametric Kruskall–Wallis test and Bonferroni post hoc analysis. Differences with $P < 0.05$ were considered statistically significant.

Results

Infarct volume

Cerebral infarct was measured by TTC staining in the area known to be the most damaged following occlusion of the middle cerebral artery. Fig. 1 shows a histogram of overall infarct volume in the four treatment groups, as well as a sample

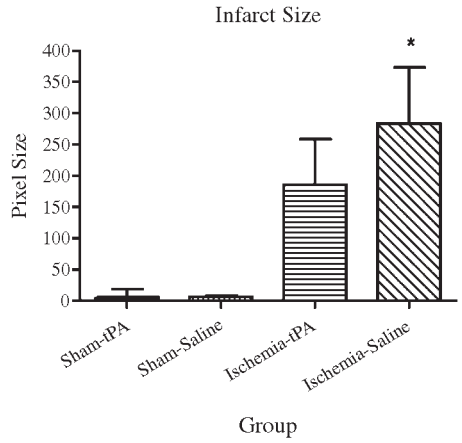
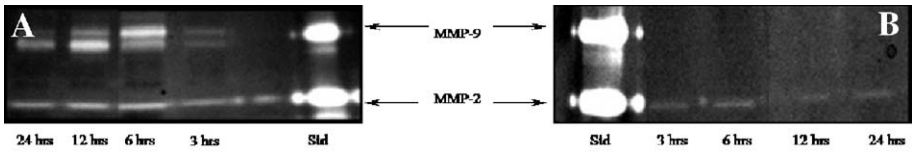


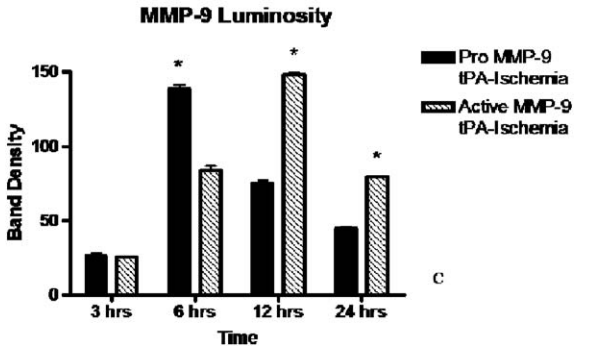
Fig. 1. Infarct volume measured by TTC staining. tPA treatment significantly reduces infarct volume when compared to saline treatment.

section obtained from this study. Overall infarct volumes were Sham-tPA: 5.80 ± 4.55 [mean ± SE]; Sham-Saline: 5.00 ± 4.23; Ischemia-tPA: 186.1 ± 73.45; Ischemia-Saline: 284.8 ± 88.74 (all $P < 0.05$) tPA significantly reduced infarct volume in ischemic animals. Although these data are widely published, it is included here to demonstrate the validity of our model (Vivien and Buisson, 2000; Yepes et al., 2003; Graham, 2003; Gautier et al., 2003).

Gelatin zymography

Results obtained from gelatin zymography experiments demonstrate an increase in MMP-9 activation at 6, 12, and 24 h following ischemia in tPA-treated animals. The pro-form of MMP-9 was increased significantly at 6 h only. These data are represented in Fig. 2A. The contralateral (non-ischemic) hemisphere of tPA-treated animals did not demonstrate any changes in either the pro- or active forms of MMP-9 at any of the time points examined (data not shown). Similarly, saline-treated ischemic animals and sham-operated animals did not demonstrate MMP-9 activation (Fig. 2B). The pro-form of MMP-9 observed in the homogenates from these groups was used as a baseline measure for any changes that occurred. These





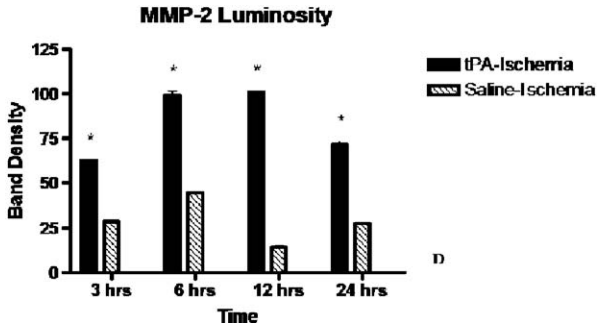


Fig. 2. Representative zymograms. (A) Zymogram shows gelatinolytic activity of tPA-treated ischemic animals at all time points. Demonstrates MMP-9 activity at 6, 12, and 24 h after ischemia, and an increase in the pro-form of MMP-9 only at 6 h following ischemia. Note that MMP-2 activation is the same at all time points. (B) Zymogram depicts gelatinolytic activity of saline-treated sham animals at all time points. This zymogram shows MMP-2 activity at all time points with the complete absence of MMP-9 activity. (C) Histogram represents band luminosity at all time points studied for MMP-9. Asterisks represent statistical significant difference when active MMP-9 is compared to pro-MMP-9. Data for the gel shown in panel B are not shown here, as MMP-9 is not present giving us a luminosity of 0. Stds—standards of human. (D) Histogram represents band luminosity for MMP-2 at all time points measured. There was no statistical significance when temporal changes in MMP-2 were measured, but activation levels differed significantly between tPA-treated animals and saline-treated animals. Asterisks mark those groups which did reach statistical significance.

results were obtained in both the cortex and striatum of all animals.

In contrast to the results obtained from MMP-9, a band depicting MMP-2 activation was observed at all time points in both treated and non-treated animals. This result was consistently obtained in both sham-operated and ischemic groups. Although this band appeared in all groups studied at all time points examined, there were no significant temporal changes observed in the activation of MMP-2. This result of MMP-2 being constitutively present is expected based upon the literature (Heo et al., 1999; Aoki et al., 2002; Asahi et al., 2000; Gasche et al., 1999, 2001a). However, a significant difference in the activity of MMP-2 between saline-treated animals and the tPA-treated animals was observed. This significant difference was again only observed in ischemic groups.

IgG extravasation

This gross measure of BBB breakdown demonstrated that non-treated animals showed IgG extravasation at all time points, with 24 h exhibiting the greatest IgG leakage from blood vessels (Figs. 3D and E). In tPA-treated animals, 12 h appeared to be the threshold for BBB integrity. Beyond this time point, blood vessels became significantly more permeable. Sham-operated animals demonstrated no leeching of IgG from cerebral blood vessels (Figs. 3A–C). Fig. 3H displays a histogram of the relative IgG staining in the parenchyma.

Immunohistochemistry

Immunostaining was performed on frozen tissue sections to measure the extent of breakdown of structural components of the BBB and to localize MMP-2 and -9 to specific cell type. Laminin and fibronectin were examined as they are substrates for the proteolytic activity of MMP-2 and -9 and are key structural components of the BBB. The analyses of immunoreacted sections from all groups revealed that laminin breakdown occurred within 6 h following ischemia in both treated and non-treated animals. The maximal amount of laminin breakdown occurred at 9 h following ischemia in treated animals and at 24 h non-treated animals. These data are shown in Fig. 4. Note the difference in the amount of laminin staining between contralateral and ipsilateral hemispheres. The ipsilateral or ischemic hemisphere demonstrates significantly less laminin immunostaining than the contralateral hemisphere. Also of note, is the morphology of the blood vessels which are present in either hemisphere. In ischemic areas, vessels have a non-continuous appearance suggesting that they have begun to lose their integrity when compared to the non-ischemic hemisphere. Fibronectin breakdown followed a similar time course as laminin breakdown in tPA-treated animals. Both laminin and fibronectin destruction were restricted to the ischemic hemisphere. Fig. 5 depicts fibronectin destruction after 9 h of ischemia in a tPA-treated animal. Non-treated animals did not demonstrate any significant destruction of fibronectin until 12 h following ischemia whereas sham-operated animals did not show any breakdown of fibronectin (Figs. 5E and F).

Localization of MMPs through immunohistochemistry showed that MMP-2 was largely but not solely, restricted to neuronal cells throughout the brain (Figs. 6A–C). This expression of MMP-2 did not change based on treatment or time point studied. MMP-2 was also observed to be present in astrocytes throughout both hemispheres in non-treated animals only, including both sham and ischemic animals. The glial expression of MMP-2 was rarely observed only in tPA-treated animals (Figs. 6D–F). This result is in concordance with a study examining MMP inhibition in superoxide-dismutase knockout mice (Gasche et al., 2001b).

Immunostaining demonstrated that MMP-9 was not expressed in either neuronal or glial cells (Figs. 5G–L). This was consistent across all treatment conditions and time points. MMP-9 immunostaining was largely detected extracellularly. This extracellular expression of MMP-9 was expected since the enzyme acts extracellularly to degrade the proteins of the extracellular matrix thereby compromising the BBB.

In situ zymography

The gelatin zymography described above does not accurately regionalize the activity of MMPs, rather it demonstrates the global MMP activity level in a tissue. Therefore, we performed an in situ gelatinolytic assay on tissue sections to measure and localize the activity of MMPs. Although this assay does not differentiate between the two gelatinases, it does provide information of the regional activation that the gelatin zymography cannot. Though we did observe a fluorescent signal in sham-operated animals, this was largely restricted to neuronal cells and blood vessels (Fig. 7). This pattern of expression closely resembled the fluorescent immunohistochemistry co-localizing MMP-2 to neurons. In ischemic animals, an increase in the intensity of the fluorescent signal was noted; this can be seen in Figs. 7D–I. No change was observed in the pattern of MMP activity. The in situ zymography consistently demonstrated neuronal activity, regardless of time point or treatment.

Discussion

In the present study, we found that tPA administration following a thromboembolic cerebral infarct induces the activation of MMP-2 and -9 and that this compromises the BBB in a time-dependent fashion. We demonstrate a significant reduction in infarct volume conferred by thrombolytic treatment with tPA. This result, widely published (Vivien and Buisson, 2000; Yepes et al., 2003; Graham, 2003; Gautier et al., 2003), confirmed by TTC staining demonstrates the reliability of the ischemic model used for this study. Use of TTC staining as a measure of infarct volume has been shown to be equivalent if not superior to cresyl violet staining and hematoxylin–eosin staining; two alternative histological techniques (Tureyen et al., 2004; Bederson et al., 1986). Our goal in the use of TTC as a measure of infarct was not to reproduce previously published reports but rather to confirm

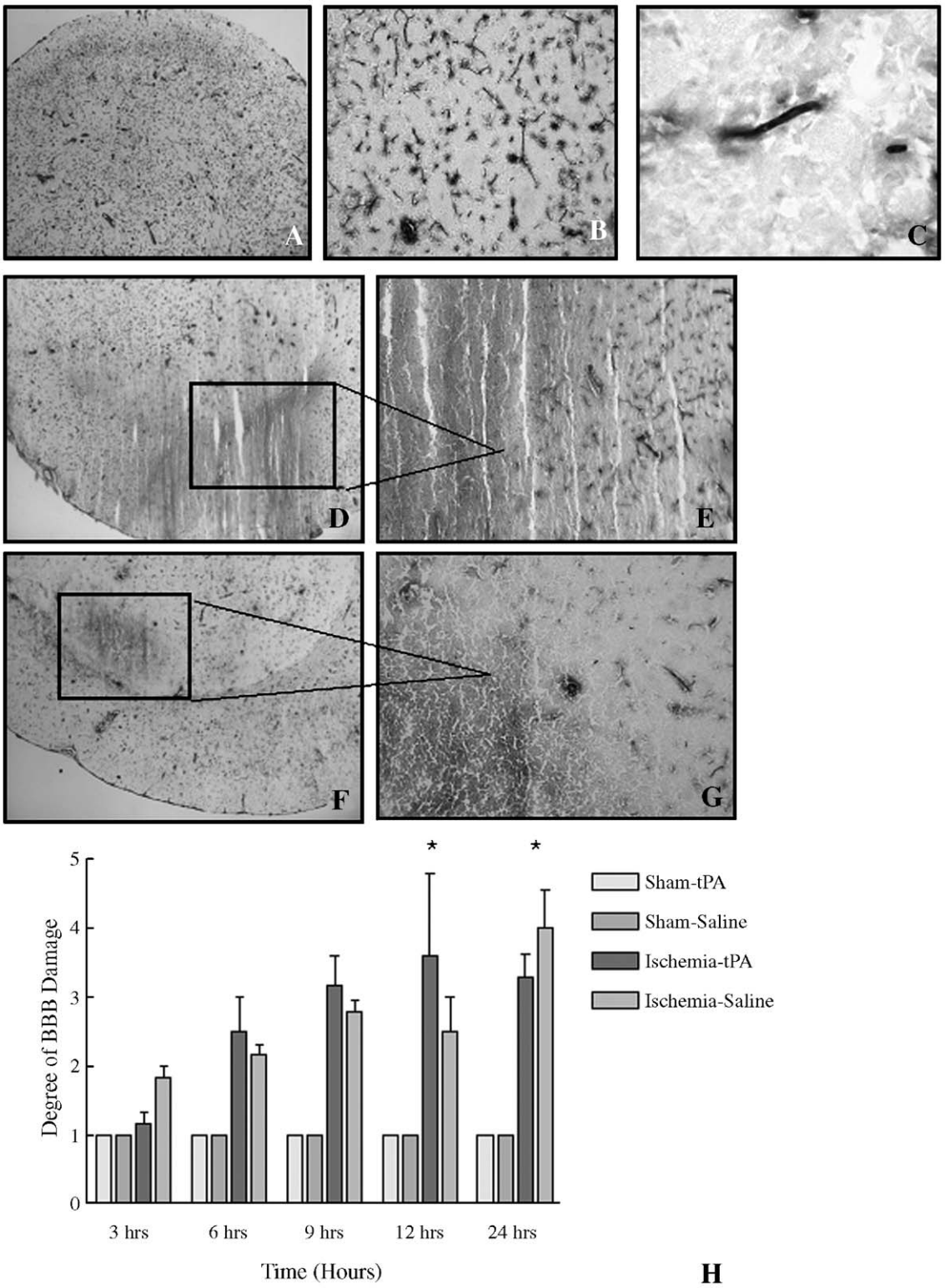


Fig. 3. IgG extravasation. (A–C) Represent a sham-saline-treated animal. Blood vessels are fully intact and IgG can be seen within them. There is no leakage of IgG to the surrounding parenchyma. (D–E) Represent a saline-treated ischemic animal after 24 h. IgG has significantly leaked into the brain parenchyma, and you can distinguish a border zone between the leaky blood vessels and those that remain intact. (F–G) Represent a tPA-treated ischemic animal after 9 h. The leakage from blood vessels at this time point is more punctate and distinct. (A, D, F) Magnification: 4×. (B, E, F) Magnification: 10×. (C) Magnification: 20×. (H) Histogram depicting the extravasation of IgG. Saline-treated ischemic animals demonstrate BBB breakdown as early as 3 h. tPA-treated ischemic animals demonstrate IgG extravasation after 6 h of ischemia.

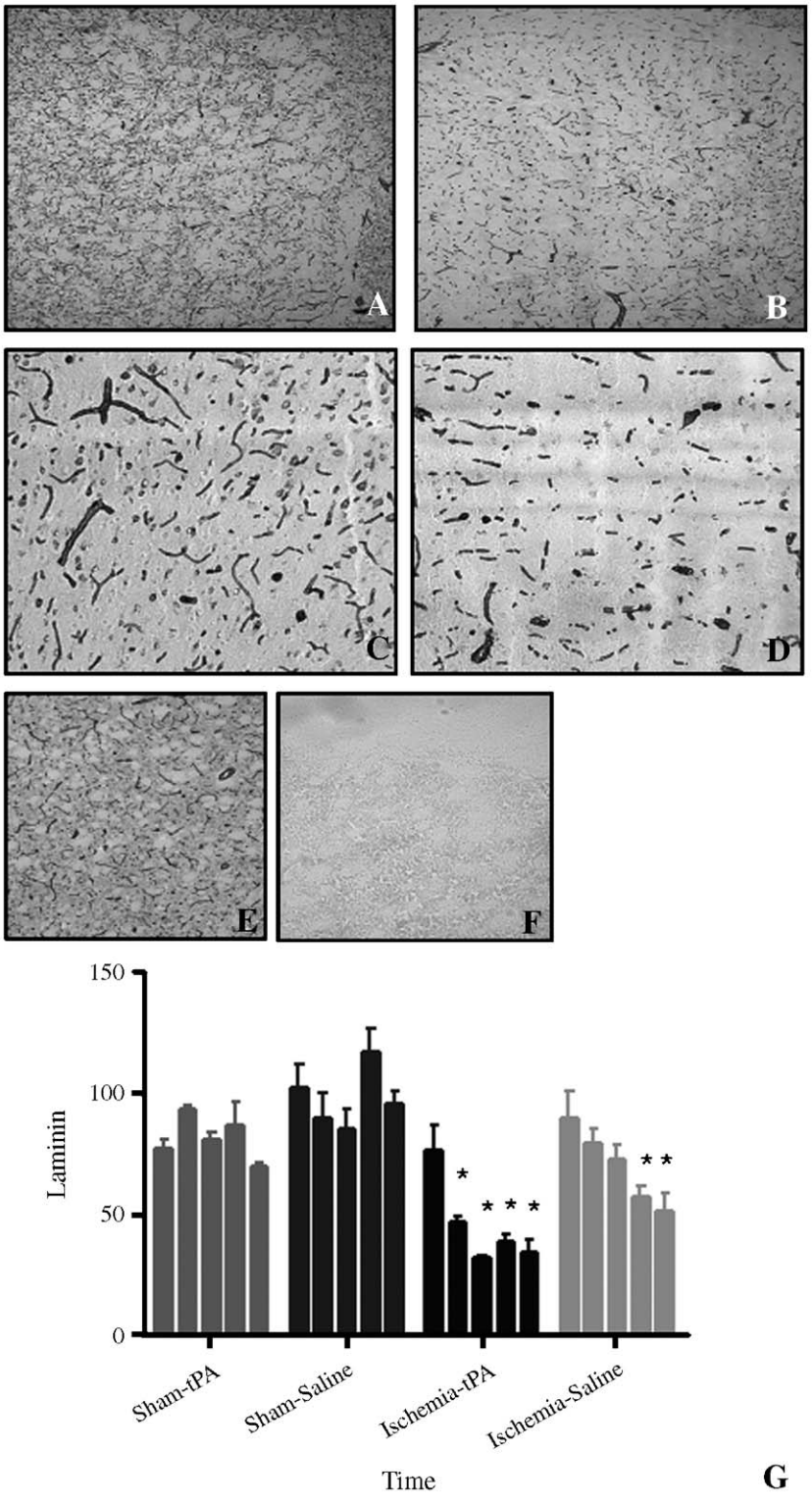


Fig. 4. Immunostaining for the structural protein laminin. (A–B) Sections obtained from the same tPA-treated ischemic animal, 6 h post-ischemia. (A) Represents the left, contralateral hemisphere. (B) Represents the right, ipsilateral ischemic hemisphere. Note the vast difference in the amount of laminin present in each of the sections. This is an indication of BBB destruction. Panels C–D are from the same saline-treated ischemic animal after 24 h. (C) Represents the left, contralateral hemisphere. (D) Represents the right, ipsilateral hemisphere. Again, note the lack of laminin staining in panel D. Also of note is the broken appearance of the blood vessels which are present. (E) Immunostaining for laminin in a tPA-treated sham animal. Note how all blood vessels appear intact. Staining shown here is representative of all tPA-treated sham animals at all time points. (F) Negative control for laminin immunostaining. Slides were treated under the same conditions with the exception of primary antibody incubation. Magnification: 10×. (G) Histogram depicting the loss of laminin immunostaining over time. Each group contains a bar representing a time point in increasing order. All ischemic animals demonstrate loss of the structural protein laminin after 6 h. In tPA-treated ischemic animals, the loss of laminin is significant compared to both sham groups at 6, 9, 12, and 24 h and significantly different from non-treated ischemic animals at 6 and 9 h. Ischemic animals treated with tPA showed more laminin loss than those animals that received saline following ischemia at the 6 and 9 h time points.

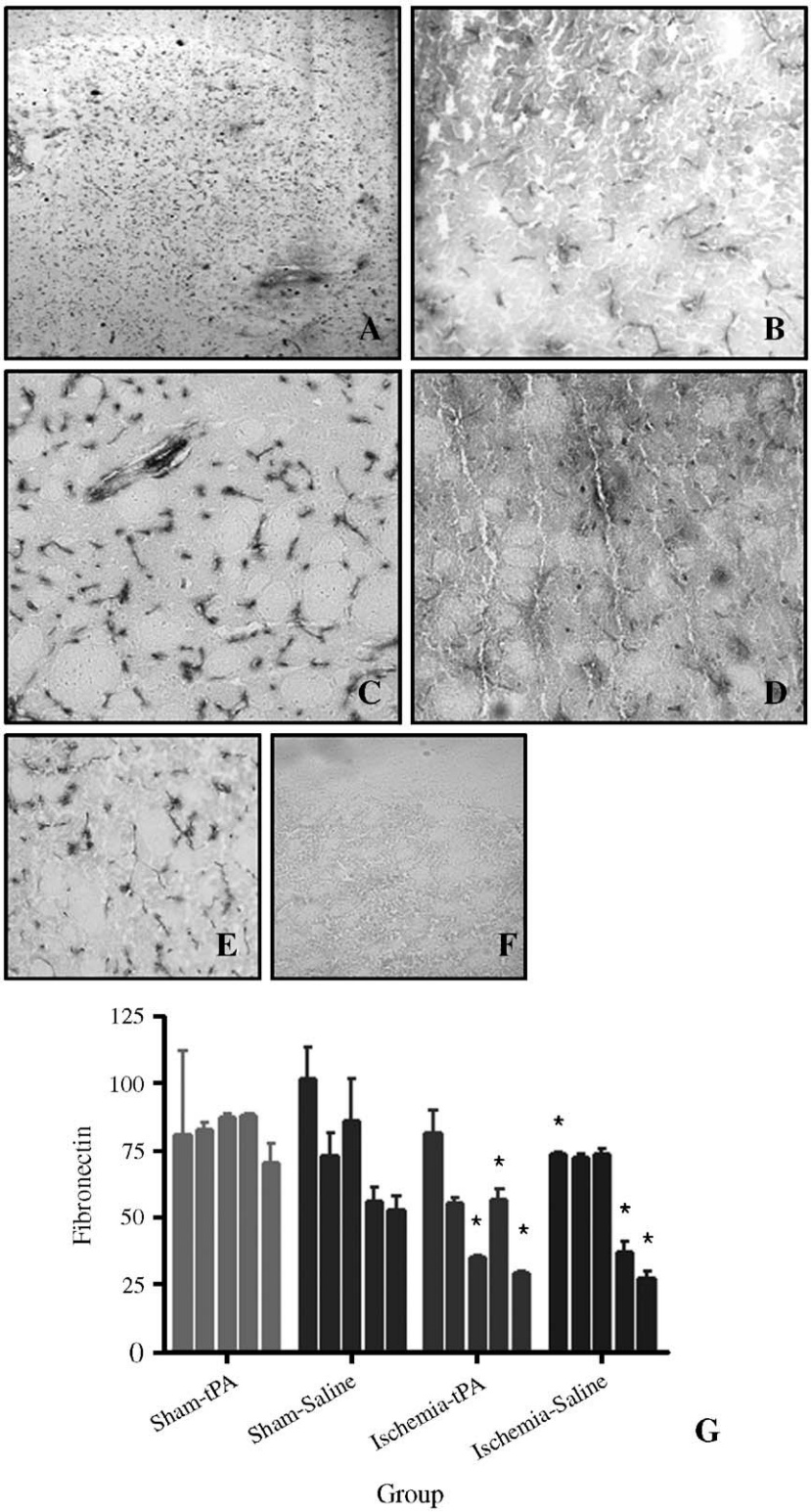


Fig. 5. Immunostaining for the structural protein fibronectin. (A–B) Figures are representative of a tPA-treated animal after 9 h of ischemia. Note the breakdown of fibronectin which resembles the leakiness of IgG in previous figures. (A) Magnification 4×. (B) Magnification 10×. (C–D) Sections are from the same animal-saline-treated, ischemic animal at 24 h. (C) Photomicrograph representative of the left, contralateral hemisphere. (D) Represents the right, ipsilateral hemisphere. Note the different patterns of staining between the two hemispheres. (E) Represents a sham, saline-treated animal. Fibronectin surrounding blood vessels is intact and there is no leakage to the surrounding parenchyma. (F) Negative control for fibronectin immunostaining. Slides underwent the same immunohistochemical protocol without the application of the primary antibody. (G) Histogram depicting the breakdown of fibronectin over time. tPA-treated ischemic animals demonstrate loss of fibronectin after only 6 h of ischemia, while non-treated animals do not demonstrate any fibronectin loss until 12 h post-ischemia. The loss of fibronectin is considered significant for tPA-treated ischemic animals compared to sham animals at 9, 12, and 24 h following ischemia. Non-treated ischemic animals showed a significant amount of fibronectin loss compared to sham animals at 3, 12, and 24 h after ischemic injury. tPA-treated ischemic animals demonstrated more loss of fibronectin than non-treated animals only at the 9 h time point.

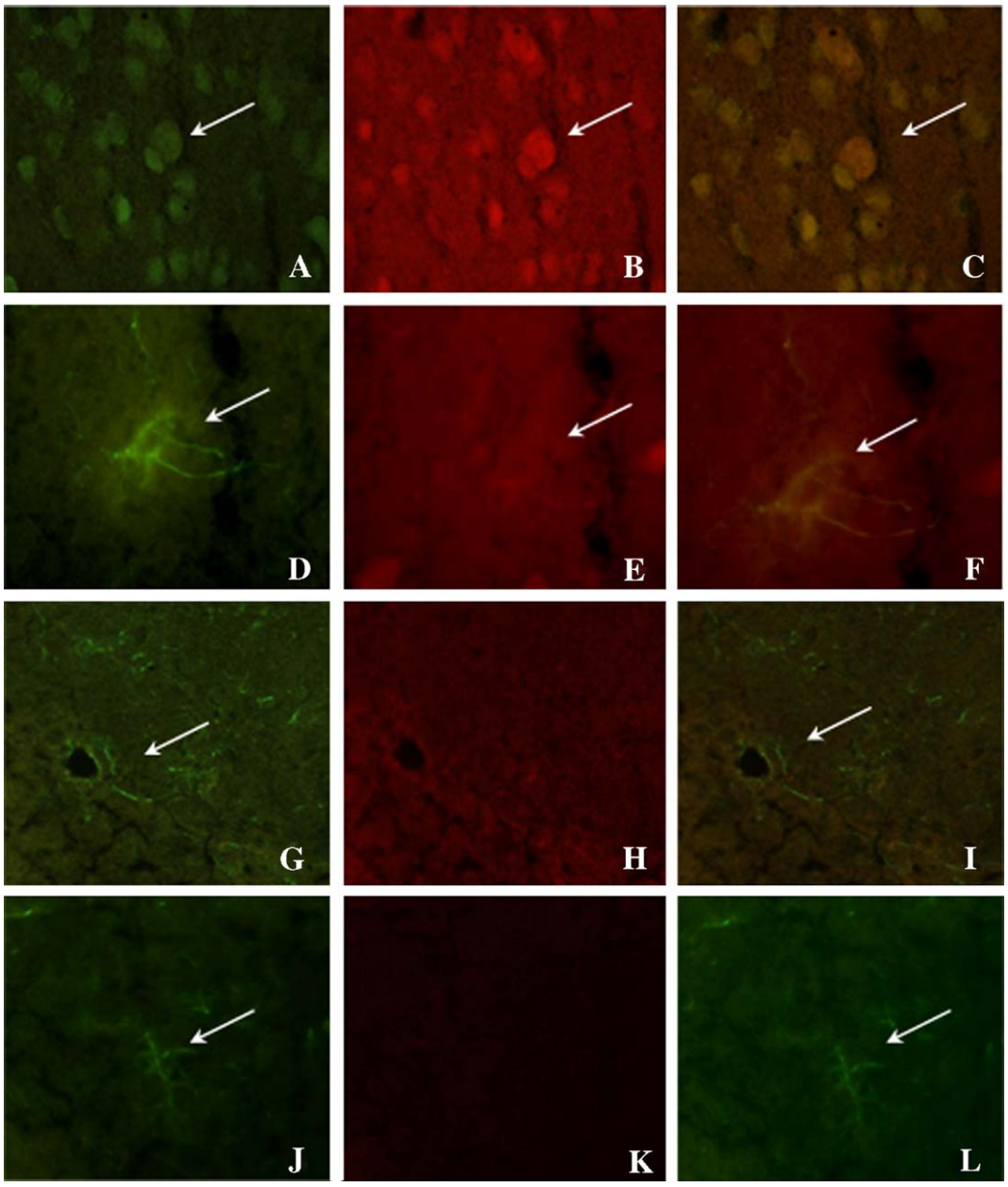


Fig. 6. Double immunofluorescent staining. (A) Depicts NeuN staining, showing neurons. (B) Depicts the immunostaining for MMP-2. (C) The merged image. This section is from a tPA-treated ischemic animal, white arrows indicate a neuronal cell body expressing MMP-2. The result shown here is consistent among all animals included in the study. MMP-2 is clearly localized to neuronal cells. (D) Depicts GFAP staining, a marker for astrocytes. (E) Depicts the immunostaining for MMP-2. (F) The merged image. Note that only one astrocyte in this area is positive for MMP-2 (white arrows). This section is from a tPA-treated ischemic animal and the lack of glial MMP-2 expression is observed in all tPA-treated animals at all time points studied. (G) Depicts immunostaining for GFAP. (H) Demonstrates immunostaining for MMP-9. (I) Overlaid image demonstrating no MMP-9 expression in astrocytes (white arrows in panels G and I). Sections are from non-treated ischemic animals (G, H, I). Panels J–L are sections obtained from tPA-treated ischemic animals. (J) Represents immunostaining for GFAP. (K) Immunostaining for MMP-9. (L) Overlaid image, again showing a lack of MMP-9 expression in astrocytes (white arrows).

the validity of our model. And indeed we observed uniform and reproducible cerebral infarcts.

Gelatin zymography experiments show a clear increase in MMP activity following tPA administration. Although it has been previously demonstrated using a similar animal model that MMP-9 was upregulated at 6 h (Aoki et al., 2002) following ischemia, our results are the first to outline the full temporal profile of the combination of tPA administration in a thromboembolic model in the first 24 h of ischemic injury. During the preparation of this manuscript, two reports were published which also outline the activation of MMP-9 following tPA administration. These manuscripts involved the use of an intraluminal suture model of ischemia followed by mechanical reperfusion, and one (Tsuji et al., 2005), also examined MMP-9 with tPA-knockout mice. The main conclusions of this study were that tPA administered exogenously

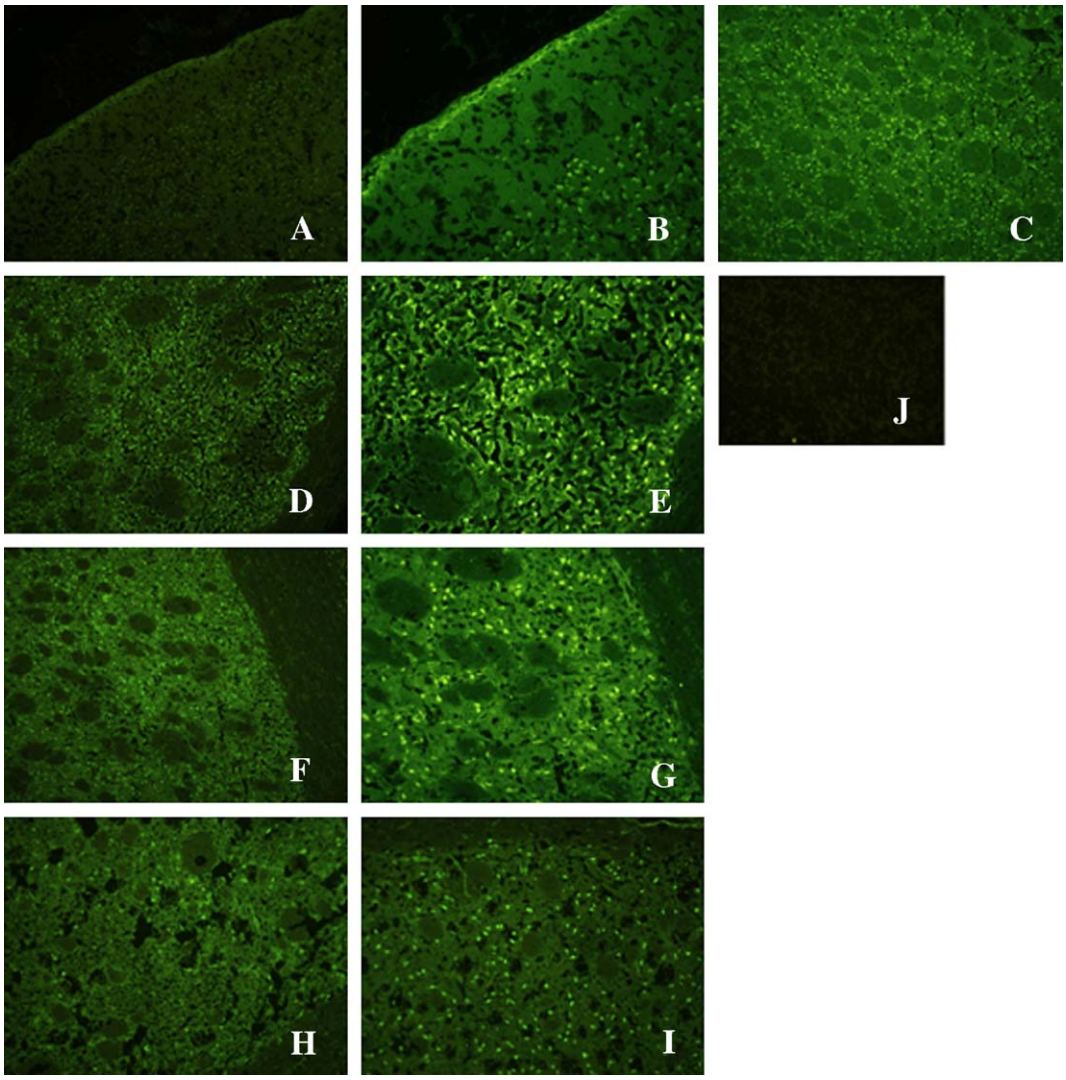


Fig. 7. In situ Zymography. Panels A–C depict sections from a sham-operated, saline-treated animal. This is representative of a baseline of MMP activation for comparison among experimental groups. Note that layers 1 and 2 of the cortex do not demonstrate any MMP activity. This restriction to cell bodies is consistent throughout all time points and treatment modalities. (A) Magnification 10×; (B) 20×; (C) 20× in the right striatum. (D–G) Depict sections through the ipsilateral ischemic striatum (D, E) and the contralateral non-ischemic striatum (F, G) of a tPA-treated ischemic animal 12 h post-ischemia. Note the difference in the pattern of staining between the two hemispheres. In the ipsilateral striatum, cells appear brighter and areas surrounding cells are more fluorescent in comparison to the contralateral striatum and to the non-ischemic sections seen in panels A–C. Panels H and I depict ipsilateral (H) and contralateral (I) striatal sections in a non-treated ischemic animal. Cells appear intact and the extracellular space does not appear to demonstrate any MMP activation as seen in panels D and E. (J) Represents a negative control for these sections. The slide was treated to all of the same conditions as test slides with the exception of incubation with the DQ-labeled gelatin.

increased MMP-9 activation twofold, and the genetic knockout of tPA reduced MMP-9 levels compared to wild-type mice (Tsuji et al., 2005). The second study (Kahles et al., 2005) examined BBB breakdown in the same ischemia–reperfusion (intraluminal suture) model, with the exception that tPA was administered intra-arterially to the MCA. This study found that the effects of tPA on the BBB were enhanced when tPA was incubated with clot material (Kahles et al., 2005). BBB breakdown and MMP activation are dynamic processes following cerebral ischemia and tPA administration, our study fully outlines the 24-h period after ischemic injury, in a model which is very similar to the clinical situation, using a clot formed from the animal's own blood and administering tPA 1 h after the insult, within the window of opportunity used in human stroke patients.

The results of our gelatin zymography experiments show a clear increase in both the pro- and active forms of MMP-9 after tPA administration. Six hours following ischemia, pro-MMP-9 was significantly higher than at any other time point studied, while the active form of MMP-9 was elevated at 6, 12, and 24 h post-ischemia. This increase in MMP-9 activity coincides with the greatest amount of loss of laminin and fibronectin in the same tPA-treated animals. tPA treatment of ischemic animals results in significantly greater MMP-2 activity than that observed in ischemic animals receiving saline treatment, although this activation showed no significant change over time. MMP-2 is constitutively bound to the cell membrane by a tissue inhibitor of metalloproteinases (TIMP) and a membrane-type metalloproteinase (MT-MMP). Due to the vulnerable nature of this gelatinase, it was observed that MMP-2 was activated at all time points, in all

treatment groups, including non-ischemic, non-treated animals. MMP-2 is not likely to be a major contributor to BBB breakdown in this setting, as sham-operated animals also demonstrated a baseline level of activity from this enzyme. Despite this, an increase in MMP-2 activity following tPA administration could aid in the activation of MMP-9. In terms of its cellular localization, MMP-2 was present most obviously in neuronal cells. Non-treated ischemic animals demonstrated astrocytic expression of MMP-2. However, tPA-treated animals rarely demonstrate astrocytic MMP-2 expression and such expression was restricted to the core of the infarct. The in situ zymography data were correlated to that obtained from immunohistochemistry of each of the MMPs. Neuronal cells predominantly demonstrated MMP activation as measured by this non-specific activity assay. Based on our double-immunostaining results, the activated gelatinase is likely to be MMP-2. It is also possible that the activity observed in these sections is due to other MMPs, as MMP-2 and -9 are not the only proteases able to digest gelatin, MMP-3 (stromelysin) and MMP-7 (matrilysin) are also capable (Gasche et al., 2001a) and further investigations are underway to elucidate the relative contributions of these and other metalloproteinases.

The results of immunohistochemical experiments also outlined the temporal profile of BBB breakdown by examining the loss of structural components laminin and fibronectin. Here, we observed a loss of BBB integrity which followed MMP-9 activation in tPA-treated ischemic animals. Although the loss of laminin and fibronectin was also observed in saline-treated ischemic animals, it was not observed until 12 h post-ischemia whereas tPA-treated ischemic animals demonstrated a loss of positive laminin staining which began as early as 6 h post-ischemia. This loss of laminin coincided with the increase of MMP-9 activity seen in gelatin zymography experiments therefore, it can be said that tPA is responsible for the loss of structural integrity by increasing MMP-9 activation. Sham animals receiving tPA intravenously did not demonstrate an increase in MMP-9 either in the pro-form of the active form, nor did they demonstrate destruction of the BBB. Furthermore, MMP-9 activation was not observed in the contralateral hemisphere of tPA-treated ischemic animals at any time point. This result can be explained by the hypothesis that tPA is only detrimental to the injured brain (Vivien and Buisson, 2000). If the exogenous application of tPA was harmful to the healthy brain, we would expect to see some activation of MMP-9, and breakdown of the BBB in tPA-treated sham-operated animals or in the contralateral, non-ischemic hemisphere of tPA-treated ischemic animals, but this was not observed here.

In conclusion, the current study demonstrates that the activation of MMP-9 is temporally similar to BBB breakdown as demonstrated by a loss of structural components laminin and fibronectin. The MMP-9 activation and time course of degradation of the BBB are restricted to those animals administered tPA following a focal embolic ischemic event. Our results are the first to demonstrate the temporal profile of activation of MMP-2 and -9 following tPA administration in thrombotic cerebral ischemia. These results emphasize that inhibition of MMP-9 could be the key to halting the destruction of the BBB and the hemorrhagic transformation associated with tPA administration.

Acknowledgments

Financial Support graciously provided by Heart and Stroke Foundation of Canada, Canadian Institutes of Health Research (CIHR), and the Davey Endowment for Brain Research.

References

Aoki, S., Mori, T., Wang, X., Lo, E., 2002. Blood–brain barrier disruption and matrix metalloproteinase-9 expression during reperfusion injury. Stroke 33, 2711–2717.

Asahi, A., Jung, J.C., del Zoppo, G.J., Fini, M.E., Lo, E., 2000. Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. J. Cereb. Blood Flow Metab. 20, 1681–1689.

Bederson, J.B., et al., 1986. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17 (6), 1304–1308.

Benchenane, K., et al., 2004. Equivocal roles of tissue-type plasminogen activator in stroke-induced injury. Trends Neurosci. 27 (3), 155–160.

Calingasan, B., Rex, Sheu, Gibson, 1995. Blood–brain barrier abnormalities in vulnerable brain regions during thiamine deficiency. Exp. Neurol. 134, 64–72.

Gasche, Y., et al., 1999. Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood–brain barrier dysfunction. J. Cereb. Blood Flow Metab. 19 (9), 1020–1028.

Gasche, C., Sugawara, Fujimura, Chan, 2001a. Matrix metalloproteinase inhibition prevents oxidative stress-associated blood brain barrier disruption after transient focal cerebral ischemia. J. Cereb. Blood Flow Metab. 21, 1393–1400.

Gasche, Y., et al., 2001b. Matrix metalloproteinase inhibition prevents oxidative stress-associated blood–brain barrier disruption after transient focal cerebral ischemia. J. Cereb. Blood Flow Metab. 21 (12), 1393–1400.

Gautier, P., Gele, P., Laprais, M., Bastide, M., Bauters, A., Deplanque, D., Jude, B., Caron, J., Bordet, R., 2003. Involvement of thrombolysis in recombinant tissue plasminogen activator-induced cerebral hemorrhages and effect on infarct volume and postischemic endothelial function. Stroke 34, 2975–2979.

Graham, G.D., 2003. Tissue plasminogen activator for acute ischemic stroke in clinical practice: a meta-analysis of safety data. Stroke 34, 2487–2850.

Heo, L., Abumiya, T., Koziol, J.A., Copeland, B.R., del Zoppo, G.J., 1999. Matrix metalloproteinase increase very early during experimental focal cerebral ischemia. J. Cereb. Blood Flow Metab. 19 (6), 624–633.

Kahles, T., et al., 2005. Tissue plasminogen activator mediated blood–brain barrier damage in transient focal cerebral ischemia in rats: relevance of interactions between thrombotic material and thrombolytic agent. Vasc. Pharmacol. 43 (4), 254–259.

Krueger, K., Busch, E., 2002. Protocol of a thromboembolic stroke model in the rat: review of the experimental procedure and comparison of models. Invest. Radiol. 37 (11), 600–608.

Lijnen, H.R., et al., 1994. Characterization of the murine plasma fibrinolytic system. Eur. J. Biochem. 224 (3), 863–871.

Paxinos, G., Watson, C., 1998. In: Paxinos, G., Watson, C. (Eds.), The Rat Brain in Stereotaxic Coordinates, 4th ed. Academic Press.

Pfefferkorn, T., Rosenberg, G.A., 2003. Closure of the blood–brain barrier by matrix metalloproteinase inhibition reduces rtPA-mediated mortality in cerebral ischemia with delayed reperfusion. Stroke 34 (8), 2025–2030.

Romanic, A.M., Madri, J.A., 1994. Extracellular matrix-degrading proteinases in the nervous system. Brain Pathol. 4 (2), 145–156.

Sumii, L., 2002. Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal cerebral ischemia in rats. Stroke 33, 831–836.

Swanson, R.A., Morton, M.T., Tsao-Wu, G., Savalos, R.A., Davidson, C., Sharp, F.R., 1990. A semiautomated method for measuring brain infarct volume. J. Cereb. Blood Flow Metab. 10, 290–293.

Todd, K.G., Butterworth, R.F., 1999. Early microglial response in experimental thiamine deficiency: an immunohistochemical analysis. Glia 25 (2), 190–198.

Tsuji, K., et al., 2005. Tissue plasminogen activator promotes matrix metalloproteinase-9 upregulation after focal cerebral ischemia. Stroke 36 (9), 1954–1959.

Tureyen, K., et al., 2004. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. J. Neurosci. Methods 139 (2), 203–207.

Vivien, D., Buisson, A., 2000. Serine protease inhibitors: novel therapeutic targets for stroke? J. Cereb. Blood Flow Metab. 20 (5), 755–764.

Wang, C.X., et al., 2001. A focal embolic model of cerebral ischemia in rats: introduction and evaluation. Brain Res. Brain Res. Protoc. 7 (2), 115–120.

Wang, X., et al., 2003. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. Nat. Med. 9 (10), 1313–1317.

Yepes, S., Moore, E.G., Bugge, T.H., Strickland, D.K., Lawrence, D.K., 2003. Tissue-type plasminogen activator induces opening of the blood–brain barrier via the LDL receptor-related protein. J. Clin. Invest. 112, 1533–1540.

Zhang, R.L., et al., 1998. Early (1 h) administration of tissue plasminogen activator reduces infarct volume without increasing hemorrhagic transformation after focal cerebral embolization in rats. J. Neurol. Sci. 160 (1), 1–8.