The tissue-type plasminogen activator–plasminogen activator inhibitor 1 complex promotes neurovascular injury in brain trauma: evidence from mice and humans

Maithili Sashindranath,1 Eunice Sales,1 Maria Daglas,1 Roxann Freeman,1 Andre L. Samson,1 Elisa J. Cops,1 Simone Beckham,1,* Adam Galle,1 Catriona McLean,2 Cristina Morganti-Kossmann,3 Jeffrey V. Rosenfeld,4 Rime Madani,5 Jean-Dominique Vassalli,5 Enming J. Su,6 Daniel A. Lawrence6 and Robert L. Medcalf1

1 Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria 3004, Australia
2 Department of Pathology, Alfred Hospital, Melbourne, Victoria 3004, Australia
3 National Trauma Research Institute, Alfred Hospital, Melbourne, Victoria 3004, Australia
4 Department of Surgery, Monash University Alfred Hospital, Melbourne, Victoria 3004, Australia
5 Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, CH-1211 Geneva 4, Switzerland
6 Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI 48109-0644, USA

*Present address: Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, VIC 3168, Australia

Correspondence to: Robert L. Medcalf, PhD, Australian Centre for Blood Diseases, Monash University, Level 6, Burnet Building, 89 Commercial Road, Melbourne 3004, Victoria, Australia
E-mail: robert.medcalf@monash.edu

The neurovascular unit provides a dynamic interface between the circulation and central nervous system. Disruption of neurovascular integrity occurs in numerous brain pathologies including neurotrauma and ischaemic stroke. Tissue plasminogen activator is a serine protease that converts plasminogen to plasm. a protease that dissolves blood clots. Besides its role in fibrinolysis, tissue plasminogen activator is abundantly expressed in the brain where it mediates extracellular proteolysis. However, proteolytically active tissue plasminogen activator also promotes neurovascular disruption after ischaemic stroke; the molecular mechanisms of this process are still unclear. Tissue plasminogen activator is naturally inhibited by serine protease inhibitors (serpins): plasminogen activator inhibitor-1, neuroserpin or protease nexin-1 that results in the formation of serpin:protease complexes. Proteases and serpin:protease complexes are cleared through high-affinity binding to low-density lipoprotein receptors, but their binding to these receptors can also transmit extracellular signals across the plasma membrane. The matrix metalloproteinases are the second major proteolytic system in the mammalian brain, and like tissue plasminogen activators are pivotal to neurological function but can also degrade structures of the neurovascular unit after injury. Herein, we show that tissue plasminogen activator potentiates neurovascular damage in a dose-dependent manner in a mouse model of neurotrauma. Surprisingly, inhibition of activity following administration of plasminogen activator inhibitor-1 significantly increased cerebrovascular permeability. This led to our finding that formation of complexes between tissue plasminogen activator and plasminogen activator inhibitor-1 in the brain parenchyma facilitates post-traumatic cerebrovascular damage.
We demonstrate that following trauma, the complex binds to low-density lipoprotein receptors, triggering the induction of matrix metalloproteinase-3. Accordingly, pharmacological inhibition of matrix metalloproteinase-3 attenuates neurovascular permeability and improves neurological function in injured mice. Our results are clinically relevant, because concentrations of tissue plasminogen activator: plasminogen activator inhibitor-1 complex and matrix metalloproteinase-3 are significantly elevated in cerebrospinal fluid of trauma patients and correlate with neurological outcome. In a separate study, we found that matrix metalloproteinase-3 and albumin, a marker of cerebrovascular damage, were significantly increased in brain tissue of patients with neurotrauma. Perturbation of neurovascular homeostasis causing oedema, inflammation and cell death is an important cause of acute and long-term neurological dysfunction after trauma. A role for the tissue plasminogen activator–matrix metalloproteinase axis in promoting neurovascular disruption after neurotrauma has not been described thus far. Targeting tissue plasminogen activator: plasminogen activator inhibitor-1 complex signalling or downstream matrix metalloproteinase-3 induction may provide viable therapeutic strategies to reduce cerebrovascular permeability after neurotrauma.

Keywords: neurovascular unit; traumatic brain injury; tissue plasminogen activator; plasminogen activator inhibitor-1; matrix metalloproteinase-3

Abbreviations: GOS-E = Glasgow outcome scale-extended; LDLRs = low density lipoprotein receptors; MMP = matrix metalloproteinase; NNGH = N-isobutyl-N-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid; PAI1 = plasminogen activator inhibitor-1; TBI = traumatic brain injury; t-PA = tissue-type plasminogen activator

Introduction

The neurovascular unit is a highly regulated structure between the interstitial space of the brain and the peripheral circulation. It consists of microvascular endothelium on the luminal surface of a basement membrane, and astrocytes, pericytes and neurons on the abluminal surface (Hawkins and Davis, 2005). Increased neurovascular permeability contributes to morbidity and mortality in several neurological pathologies (Hawkins and Davis, 2005). The disruption of neurovascular integrity is not only a consequence of pathology such as in traumatic brain injury (Shlosberg et al., 2010; Zink et al., 2010) and ischaemic stroke (Su et al., 2008; Yang and Rosenberg, 2011) but also may be the precipitating event such as in multiple sclerosis (Zlokovic, 2008; Alvarez et al., 2011).

TBI is the leading cause of death and disability in individuals under 45 years of age. The primary phase of TBI is characterized by direct mechanical impact causing tissue damage, increased cerebrovascular permeability and impaired cerebral blood flow. The secondary phase involves inflammation, free radical generation and metabolic disturbances that precipitate further cerebrovascular disruption and neuronal loss and can lead to disability or even death (Verma, 2000). The progression of the secondary phase of trauma over days to weeks after the initial insult offers a valid time window for therapeutic intervention. However, clinical trials targeting these secondary events have been unsuccessful owing to the pathophysiological heterogeneity of trauma (Xiong et al., 2009). The neurovascular unit is comprised in ~70% of trauma cases and the extent of neurovascular permeability correlates with that of cortical dysfunction (Tomkins et al., 2011). Additionally, neurovascular disruption is linked to several long-term debilitating consequences of neurotrauma including Alzheimer’s disease and post-traumatic epilepsy (Shlosberg et al., 2010). Hence, preserving function of the neurovascular unit after TBI is likely to significantly improve clinical outcome.

Tissue-type plasminogen activator (t-PA) is a serine protease that cleaves plasminogen to generate plasmin. t-PA, commonly used as a thrombolytic agent to remove fibrin blood clots, has been directly implicated in neurovascular breakdown in ischaemic stroke, but the mechanism remains unclear (Zaheer et al., 2011). In the brain, t-PA is inhibited by one of three serine protease inhibitors (serpins): plasminogen activator inhibitor-1 (PAI1), neuroserpin and protease nexin-1, with neuroserpin being the most abundant (Vivien and Buisson, 2000). Serpins inactivate t-PA by forming stable covalent complexes that are rapidly cleared by endocytosis through low-density lipoprotein receptors (LDLRs) (Lillis et al., 2008). However, LDLRs can also transmit extracellular signals across the plasma membrane. Reports have indicated that t-PA acts as a signal transducer by binding to LDLR-related protein 1 and transcriptionally regulating genes including members of the matrix metalloproteinase (MMP) family (Herz, 2001; Candelario-Jalil et al., 2009). It is also known that free t-PA binds to LDLRs with ~300-fold lower affinity than t-PA-serpin complexes suggesting differential signalling capabilities of free and complexed t-PA (Nykjaer et al., 1994).

We have previously shown that t-PA activity is increased by ~30% within 1–3 h post-TBI, and returns to baseline levels by 24 h post-TBI in mice (Sashindranath et al., 2011). Here, we identify a novel mechanism that links t-PA with altered neurovascular permeability following TBI, whereby formation of the t-PA:PAI1 complex in vivo activates downstream signalling processes leading to induction of MMP3. MMP3 is the effector arm of this change in cerebrovascular permeability since selective inhibition of MMP3 reverses neurovascular permeability after TBI. Importantly, we show that levels of t-PA:PAI1 complex and MMP3 are significantly elevated in CSF of patients with severe TBI and these levels correlate positively with neurological outcome.

Our data suggest that targeting signalling of the t-PA:PAI1 complex and MMP3 induction will mitigate trauma-induced neurovascular degradation and hence provide viable treatment strategies for minimizing long-term consequences of trauma.
Materials and methods

Mice

All animal procedures were undertaken in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Experiments were performed with adult male mice on the C57Bl6-J background aged 8–16 weeks (mean age ± SD = 11.9 ± 1.9 weeks) and approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee. Mice were maintained under specific pathogen-free facilities at a maximum of six per cage, on a 12 h light/dark cycle and had *ad libitum* access to food and water. T4 mice are transgenic mice that constitutively produce increased levels of mouse t-PA in post-natal neurons under the control of the Thy1.2 gene promoter (Madani et al., 1999). T4 transgenic mice and their wild-type littermate controls (i.e. T4 wild-type mice) were sourced from a heterozygote breeding colony maintained at AMREP Animal Services. t-PA−/− mice were obtained from a homozygote breeding line and backcrossed 13 generations; wild-type mice were crossed with t-PA−/− mice and then backcrossed over two generations. Animals were randomly assigned to trauma/sham or treatment/vehicle groups; experiments were generally performed in the mornings and unless otherwise specified, animals were euthanized at 3 h post-surgery. Following surgery, all animals were housed individually in cages fitted with dividers and split-feeders. Animals were monitored regularly and there were no adverse events related to the trauma procedure.

Controlled cortical impact model of traumatic brain injury

TBI was induced using the controlled cortical impact model as described (Sashindranath et al., 2011). Following anaesthesia with 2,2,2-tribromoethanol (0.5 mg/kg; Sigma–Aldrich) mice were placed in a stereotaxic frame (Kopf). A surgical incision was made, the skull was exposed and a 3-mm diameter circular craniotomy was performed in a stereotaxic frame (Kopf). A surgical incision was made, the skull was exposed and a 3-mm diameter circular craniotomy was performed 

Intraventricular injections

Mice were anaesthetized and placed in a stereotaxic frame as described above. An incision was made, the skull was exposed and a small burr hole was created over the left ventricle at coordinates anterior–posterior = −0.3, mediolateral = +1.0, dorsoventral = −2.0 from bregma. A volume of 1.5 μl of t-PA:PAI1 complex (0.75 pmol; 500 nM) or vehicle (0.4 M HEPES; 0.1 M NaCl, pH 7.4) was injected over a 5-min period using a 5 μl Hamilton syringe fitted with a 28 gauge needle and once delivered, the needle was left in place for a further 5 min to minimize backflow.

Lesion volume analysis

Mice were perfused with 4% phosphate-buffered formalin, pH 7.3, at 24 h post-TBI, the whole brain was post-fixed in phosphate-buffered formalin for 24 h and then frozen on a slurry of dry ice and isopentane. Twenty micrometre sections were subsequently prepared using a cryostat. Every 10th section was stained with haematoxylin and eosin. Photomicrographs were captured using an Olympus BX50 microscope and lesion area was determined using the UTHSCSA ImageTool software (v3; University of Texas Health Science Centre at San Antonio). Tissue swelling in the injured side was accounted for by dividing the lesion area from each section by the ratio of the areas of the injured relative to injured side. The Cavalieri formula was used to calculate total lesion volume [Volume = Σ(A × f × ISF)] where A = sum of the corrected lesion areas; f = section thickness (20 μm) and ISF = inverse of the sampling fraction (1 in 10 sections was counted, i.e. sampling fraction = 1/10). For T4 transgenic and T4 wild-type injured n = 8 and sham, n = 6.

Assessment of neurovascular integrity

The extent of neurovascular permeability was assessed by Evan’s blue extravasation or by quantitating levels of albumin extracted from the cortical tissue. Evan’s blue (0.2 g/kg; Sigma–Aldrich) was injected intravenously at 2 h post-TBI. At 3 h post-trauma, following transcardial perfusion, ipsilateral and contralateral cortices were dissected, weighed and homogenized in PBS to 400 mg/ml (wet weight of tissue/volume of PBS). Proteins were then precipitated with trichloroacetic acid and the presence of Evan’s Blue in the supernatant...
quantitated spectrophotometrically by measuring optical density at 620 nm wavelength (OD620). Data are expressed as (ipsilateral [OD620 – average of OD500 and OD740])/mg wet weight of tissue) – (Contralateral [OD620 – average of OD500 and OD740])/mg wet weight of tissue). The following groups were analysed: T4 mice (injured n = 5, sham n = 4); T4 wild-type mice (injured n = 8, sham n = 3); wild-type mice (injured n = 6, sham n = 4); t-PA −/− mice (injured n = 7, sham n = 8).

We have determined that the degree of albumin extravasation correlates with that of Evan’s blue (not shown), which when injected intravenously binds to albumin and extravasates due to increased blood–brain barrier permeability. For albumin ELISAs, mice were perfused with PBS at 3 h post-trauma and the ipsilateral and contralateral cortices were harvested as described (Sashindranath et al., 2011). Tissue albumin content in the brain was determined using the Mouse Albumin ELISA Quantitation Set (Bethyl Laboratories) according to the manufacturer’s instructions. Total protein was quantitated using the BCA™ protein assay (Pierce). The amount of albumin (ng) per microgram protein in each sample was calculated from the standard curve. The difference in albumin in the ipsilateral and contralateral cortex was calculated for each animal.

### Amidolytic assay for brain-derived tissue-type plasminogen activator

T-PA activity was determined in mouse brain lysates using the amidolytic assay as described previously (Sashindranath et al., 2011).

### Gelatin zymography

Lysates were prepared of the ipsilateral and contralateral cortices and 60 µg protein from each sample was subjected to SDS–PAGE under non-reducing conditions in 10% gels containing 0.15% gelatin. After extensive washing in 2.5% Triton™ X-100, gels were incubated at 37°C overnight in a buffer containing 0.15 M NaCl, 0.05 M Tris–HCl pH 7.4 and 1% Triton™ X-100, after which the gels were subjected to Coomassie staining. Gelatinolytic activity was detected as clear bands on the Coomassie stained gel. Gelatinolytic activity was detected as clear bands on the Coomassie stained gel. T4 transgenic and T4 wild-type mice (injured n = 6); sham T4 transgenic mice (n = 4) and T4 wild-type mice (n = 3) were tested. Ventricular CSF samples were analysed similarly; a volume of 30 µl of undiluted CSF was tested.

### Fluorescein isothiocyanate–gelatinolytic assay

The EnzChek® Gelatinase Assay kit (Life Technologies) was used to measure gelatinolytic activity in lysates (wild-type mice; n = 5), according to the manufacturer’s instructions. Briefly, lysates were centrifuged at 13 000 g for 2 min and 50 µl of supernatant was incubated with the assay buffer and the fluorogenic substrate DQ™ gelatin (Life Technologies; final concentration = 100 µg/ml) overnight at 37°C. Proteolytic cleavage of DQ™ gelatin releases a green fluorescence that was measured using the FLUOstar Optima microplate reader (BMG LabTech); excitation: 495 nm, emission 515 nm.

### Assessment of matrix metalloproteinase-3 in mouse brain lysates

MMP3 content was quantitated in the same lysates used for albumin ELISAs, with the Mouse Total MMP3 DuoSet (R&D Systems) according to the manufacturer’s instructions. The difference in MMP3 concentrations in the ipsilateral and contralateral cortex was calculated for each animal.

### Matrix metalloproteinase-3 inhibition in vivo

The MMP3 inhibitor NNGH ([N-isobutyl-N-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid; Ki = 130 nM; Biomol]) was dissolved in 100% dimethyl sulphoxide (DMSO) to a final concentration of 30 mg/ml (w/v), diluted 1:4 in sterile distilled water and stored at −20°C until use. NNGH or vehicle (25% DMSO diluted in sterile distilled water) was administered through gavage at a dose of 94.8 µmol/kg at 30 min prior to and 90 min post-TBI in wild-type mice (n = 10). Mice were transcardially perfused at 3 h post-trauma as described above.

### Functional assessments

Rotarod testing (Panlab/Harvard Apparatus) was performed on animals at the 24 h time-point. Mice were first pre-trained on the Rotarod for three to four trials each at least twice within a 48 h period prior to TBI and the time taken for the mouse to fall from the Rotarod (latency) for each of the three to four trials on the day of injury was recorded (usually ~150 s). The Rotarod testing was repeated on the mice 24 h post-TBI and the ratio of the average latency values pre- and post-TBI for each mouse were determined: injured T4 transgenic and T4 wild-type mice (n = 8), sham T4 mice (n = 4), T4 wild-type mice (n = 3).

Treadmill gait analysis was performed using the DigiGait™ (Mouse Specifics Inc.) system. Digital images of paw placement were recorded through a clear treadmill from the ventral plane of the animal. Mice were first tested in a single session at a 15 cm/s treadmill speed prior to injury. At 170 min post-injury, mice were tested again at the same speed and videos of ~5 s duration of both sessions were analysed using the DigiGait™ analysis software v.11.5 (Mouse Specifics Inc.). Ratios of post:pre-injury values for each parameter of gait were then calculated.

Details of patients and CSF sample collection are provided in the online Supplementary material.

### Assessment of tissue-type plasminogen activator–plasminogen activator inhibitor-1 complex and matrix metalloproteinase-3 in cerebrospinal fluid samples

Presence of t-PA:PAI1 complex in undiluted CSF obtained within 24 h of admission was determined using the Human PAI1 t-PA complex antigen assay ELISA kit (Molecular Innovations) according to the manufacturer’s instructions. All samples were tested in duplicate. Total MMP3 in undiluted CSF (assayed in duplicate) was quantitated using the Quantikine® Human Total MMP3 ELISA kit (R&D systems Inc.) according to the manufacturer’s instructions. Glasgow outcome scale-extended (GOS-E) score 1–2 (n = 7); GOS-E score 3–4 (n = 10); GOS-E score 5–8 (n = 6); non-TBI, n = 8.
Patients and sample collection: human post-mortem brain tissue

All procedures were conducted in accordance with the Australian National Health and Medical Research Council’s National Statement on Ethical Conduct in Human Research (2007), the Victorian Human Tissue Act 1982, the National Code of Ethical Autopsy Practice, and the Victorian Government Policies and Practices in Relation to Post Mortem. Ethics approval was obtained from the Monash University Human Research Ethics Committee (MUHREC). Trauma brain samples from 12 individuals aged between 26 and 72 years (mean 51.4 years) were obtained from the Australian Brain Bank Network. The causes of injury included motor vehicle, motorcycle and cycling accidents and falls. The post-mortem intervals varied between 40 and 129 h (mean 72.8 h). Patients were grouped into two categories based on survival time post-injury as follows: survival time < 17 min (n = 6) and survival time between 6 and 93 h (mean 37.6 h; n = 6). Frontal cortical tissue was sampled. Control brain samples of 10 individuals, aged between 64 and 84.9 (mean 71.9 years), with no history of brain trauma or other neurological or psychiatric disorder and no significant neuropathology were also obtained from the Australian Brain Bank Network. The post-mortem intervals varied between 10 and 71 h (mean 45.6 h). Clinical information and epidemiological details of all patients are included in Table 1.

Sample preparation

Fresh frozen brain cortex samples were homogenized at 150 mg wet weight per millilitre of PBS + 1% Triton™ X-100 + protease and phosphatase inhibitors. Lysates were stored at −80°C until further analysis.

Quantification of albumin and matrix metalloproteinase-3 in human cortical samples from controls and patients with traumatic brain injury

Tissue homogenates were centrifuged at 13,000 g for 2 min. Albumin was determined in samples diluted in PBS, using the Human Albumin ELISA Quantitation Set ELISA kit (Bethyl Laboratories) according to the manufacturer’s instructions and assayed in duplicate. Total MMP3 in undiluted brain lysates was quantitated using the Quantikine® Human Total MMP3 ELISA kit (R&D systems Inc.) according to the manufacturer’s instructions. Individuals who died < 1 h post-trauma n = 6; individuals who survived ≥ 6 h post-trauma n = 6; non-TBI controls n = 10.

Statistical analyses

Normality tests were conducted for each experiment, and parametric or non-parametric analyses were carried out as specified below.

The student’s t-test (unpaired, two-tailed) was used to analyse differences amongst cohorts in Figs 2A(i) and 2B, 3A, B, B(iii) and C, and 4. In Figs 2A(ii) and B(ii); 3C; and 4B and C, statistical significance was determined by the student’s t-test (unpaired, two-tailed) while comparing the raw data in each cohort; although the data have been depicted as fold change relative to contralateral/vehicle control.

The Mann–Whitney test (two-tailed) was used to compare cohorts in Fig. 3B(ii).

Results

Tissue-type plasminogen activator promotes traumatic brain injury severity and increases expression of matrix metalloproteinases

To define the relationship between endogenous t-PA and the integrity of the neurovascular unit after TBI, we induced TBI in t-PA−/− mice and in transgenic T4 mice that over-express neuronal t-PA (Madani et al., 1999). T4 mice displayed the greatest degree of neurovascular permeability at 3 h post-TBI compared to their wild-type littermate controls whereas t-PA−/− mice were
significantly protected from neurovascular breakdown [Fig. 1A; T4 mice (injured n = 5, sham n = 4); T4 wild-type mice (injured n = 8, sham n = 3); wild-type mice (injured n = 6, sham n = 4); t-PA/C0/C0 mice (injured n = 7, sham n = 8)]. T4 mice also have significantly larger lesion volumes [Fig. 1B; T4 transgenic and T4 wild-type injured (n = 8), sham (n = 6)]. T4 mice have increased Rotarod deficit relative to T4 wild-type at 24 h post-severe TBI; injured T4 transgenic and T4 wild-type mice (n = 8), sham T4 mice (n = 4), T4 wild-type mice (n = 3). (D) Gelatinolytic activity is increased in T4 mice relative to T4 wild-type mice at 3 h post-TBI. (i) A t-PA-dependent increase in pro-MMP9 in the ipsilateral (ipsi) relative to the contralateral cortex (contra) in injured T4 and T4 wild-type mice; MMP-2 activity is increased equally in both T4 and T4 wild-type ipsilateral cortex; MMP2/9 positive control is conditioned media from HT1080 fibrosarcoma cells. (ii) Fold change in pro-MMP9 activity i.e. increase in activity in injured normalized to the baseline activity value of sham mice; T4 transgenic and T4 wild-type mice (injured n = 6); sham T4 (n = 4), T4 wild-type (n = 3). (E) Temporal profile of gelatinolytic activity shows a selective increase at 3 h following TBI and return to baseline levels by 24 h post-TBI in the ipsilateral relative to the contralateral cortex as determined by extent of cleavage of fluorescein isothiocyanate–gelatin in wild-type mice; n = 5. Data expressed as mean ± SD. T4-Wt = T4 wild-type.

Blockade of tissue-type plasminogen activator activity with plasminogen activator inhibitor-1 enhances neurovascular permeability

As we have previously shown (Sashindranath et al., 2011), endogenous t-PA activity is increased in the ipsilateral cortex within area. However, MMP3 messenger RNA expression was significantly increased in the ipsilateral cortex in all animals except for t-PA/C0/C0 mice (not shown). Total gelatinolytic activity was elevated in the ipsilateral cortex of wild-type mice after 3 h post-TBI, which returned to basal levels by 24 h (Fig. 1E; wild-type mice; n = 5). Interestingly, t-PA activity is similarly increased from 1 to 3 h post-TBI and returns to baseline levels by 24 h (Sashindranath et al., 2011) suggesting that endogenous t-PA is associated with the induction of MMPs after TBI.
To support this finding and to rule out the possibility that t-PA−/− mice were inherently protected against further injury, the preformed t-PA:PAI1 complex was injected intracortically into t-PA−/− mice after TBI. Injection of the t-PA:PAI1 complex caused a significant increase in albumin extravasation [Fig. 3B(ii); n = 10 t-PA:PAI1 complex, n = 9 vehicle] at 3 h post-TBI. A 5-fold increase in MMP3 messenger RNA levels [Fig. 3B(ii); n = 4 t-PA:PAI1 complex, n = 5 vehicle] as well as a significant induction in MMP3 antigen was also observed [Fig. 3B(iii); n = 10 t-PA:PAI1 complex, n = 9 vehicle] in the ipsilateral cortex. Injection of the t-PA:PAI1 complex also worsened neurological function as measured by changes in gait indices using the DigiGait™ system (Fig. 4C; n = 10). Hence, t-PA:PAI1 complex formation is sufficient to increase permeability of the neurovascular unit and impair motor function post-TBI.

**The tissue-type plasminogen activator–plasminogen activator inhibitor-1 complex increases albumin extravasation into the brain parenchyma in the absence of injury**

A non-injury paradigm was next used to determine whether the t-PA:PAI1 complex could directly promote cerebrovascular permeability. As shown in Fig. 3D, intraventricular injection of t-PA:PAI1 complex in uninjured wild-type mice increased albumin extravasation in the contralateral hemisphere 1 h post-injection (n = 5 t-PA:PAI1 complex, n = 4 vehicle). Hence the t-PA:PAI1 complex is sufficient to alter permeability of the neurovascular unit in the absence of injury.

**Inhibition of matrix metalloproteinase-3 attenuates traumatic brain injury severity**

The t-PA-dependent induction of MMPs following TBI prompted us to determine whether inhibition of MMP activity would rescue trauma-induced neurovascular dysfunction. Administration of the MMP2/9 inhibitor SB-3CT (Gu *et al*., 2005) produced a non-significant decrease in albumin extravasation at 3 h post-TBI (not shown). However, administration of the orally active MMP3 inhibitor NNGH (MacPherson *et al*., 1997) at 30 min prior to and 90 min after induction of TBI in wild-type mice significantly reduced neurovascular damage (Fig. 4A; n = 10) and also improved neurological outcome at 3 h post-TBI as assessed by the DigiGait™ system (Fig. 4C and Supplementary Video 1; n = 9). Administration of NNGH did not alter the TBI-induced increase in t-PA activity (Fig. 4B; n = 10). Hence MMP3 is downstream of t-PA:PAI1-mediated signalling through LDLRs and is a key modulator of the enhanced neurovascular permeability.

**Tissue-type plasminogen activator–plasminogen activator inhibitor-1 complex and matrix metalloproteinase-3 levels are elevated in the cerebrospinal fluid of patients with traumatic brain injury**

To determine the clinical relevance of these experimental findings, we examined levels of MMP3 and t-PA:PAI1 complex in CSF from 23 human patients with TBI categorized into three groups based on the severity and outcome of the trauma as determined by the GOS-E score. A GOS-E score of 1 indicates
mortality and a score of 8 indicates sufficient recovery. The concentrations of t-PA:PAI1 complex were significantly elevated in patients with TBI with GOS-E scores 1–2, slightly lower in patients with a GOS-E scores 3–4 and absent in patients with GOS-E scores of 5–8, as well as in the eight non-TBI controls that were included in the study [Fig 5A(i)]. Therefore, t-PA:PAI1 complex levels were significantly elevated in patients with TBI and positively correlated with neurological outcome (i.e. negatively correlated with GOS-E score; \( r = -0.539; P = 0.0079 \); Supplementary Fig. 1A). Importantly, serum t-PA:PAI1 complex levels did not correlate with GOS-E score or with CSF t-PA:PAI1 complex levels (Supplementary Fig. 1C and D; \( n = 9 \)). However, due to the small dataset and the absence of corresponding CSF:serum albumin quotient data, we are unable to conclude whether the presence of the t-PA:PAI1 complex in the CSF of patients with severe TBI is due to altered blood–brain or blood–CSF barrier integrity or if derived from the CNS. Fibrin zymography, which detects active and complexed t-PA (Philips et al., 1984), also revealed the presence of high-molecular weight t-PA complexes with little or no free t-PA in these CSF samples (not shown). Concentrations of MMP3 antigen was also significantly increased in the CSF of patients with severe TBI, while in the CSF of patients with less severe TBI, MMP3 concentrations were similar to that of non-TBI cases [Fig. 5A(ii)] and correlated positively with that of the t-PA:PAI1 complex (\( r = 0.585; P = 0.0006 \); Supplementary Fig. 1B) in all cases; GOS-E score 1–2 (\( n = 7 \)); GOS-E score 3–4 (\( n = 10 \)); GOS-E score 5–8 (\( n = 6 \)); non-TBI \( n = 8 \).
Gelatin zymography also revealed that MMP-9 and most likely MMP-9:TIMP-1 (tissue inhibitor of metalloproteinase-1) complex levels were markedly elevated in the CSF of all patients with TBI as compared with non-TBI control patients (Fig. 5B). These data confirm a preliminary report suggesting that MMP3 and MMP-9 concentrations in CSF are increased with severity following TBI (Grossetete et al., 2009).

In a separate cohort of post-mortem cortical tissue of human TBI cases and non-TBI controls, significant increases in albumin and MMP3 antigen were seen in samples from patients who survived ≥6 h post-injury (Fig. 5C); patients who died within 1 h of injury (n = 6), patients who survived ≥6 h post-injury (n = 6), non-TBI control cases (n = 10). Therefore, MMP3 may also be involved in the secondary phase of blood–brain barrier opening after brain injury (Yang and Rosenberg, 2011). To our knowledge, this is the first report showing increased MMP3 expression in post-mortem tissue of human TBI cases. We were unable to detect the t-PA:PAI1 complex in these post-mortem samples by ELISA, although others have shown increased astrocytic and microglial expression of both t-PA and PAI1 in post-mortem human trauma brain tissue (Dietzmann et al., 2000).

Matrix metalloproteinase-3 and albumin are significantly increased in human autopsy brain tissue of patients with neurotrauma

In a separate cohort of post-mortem cortical tissue of human TBI cases and non-TBI controls, significant increases in albumin and MMP3 antigen were seen in samples from patients who survived ≥6 h post-injury (Fig. 5C); patients who died within 1 h of injury (n = 6), patients who survived ≥6 h post-injury (n = 6), non-TBI control cases (n = 10). Therefore, MMP3 may also be involved in the secondary phase of blood–brain barrier opening after brain injury (Yang and Rosenberg, 2011). To our knowledge, this is the first report showing increased MMP3 expression in post-mortem tissue of human TBI cases. We were unable to detect the t-PA:PAI1 complex in these post-mortem samples by ELISA, although others have shown increased astrocytic and microglial expression of both t-PA and PAI1 in post-mortem human trauma brain tissue (Dietzmann et al., 2000).

Discussion

The neurovascular unit is a complex structure that defines the operating environment of the CNS by modulating the passage
of substances between the blood and the CSF and brain parenchyma (Hawkins and Davis, 2005). The plasminogen activating system and t-PA in particular is critical for extracellular proteolysis in the brain, which regulates cell migration, neurite extension and synaptic plasticity. The molecular basis for the adverse effects of t-PA on neurovascular homeostasis has remained inconclusive (Lo et al., 2004; Lemarchant et al., 2012). Indeed, various models of t-PA-mediated exacerbation of neurovascular permeability have been described thus far (Yepes et al., 2003; Polavarapu et al., 2007; Zhang et al., 2009b; Macrez et al., 2011). In models of cerebral ischaemia, t-PA promotes haemorrhagic transformation by cleaving latent platelet-derived growth factor-CC (PDGF-CC) through LDLR-related protein, thereby activating the PDGF/C11 receptor (Su et al., 2008). The binding of t-PA to LDLR-related protein has been shown to increase neurovascular permeability through both MMP9-dependent (Tsuji et al., 2005; Zhang et al., 2009a) and independent means (Yepes et al., 2003). Proteolytically active t-PA also induces MMP3 through LDLRs and the NFκB pathway in endothelial cells under ischaemic stress (Suzuki et al., 2009), which is linked to increased bleeding in a model of intracranial haemorrhage (Suzuki et al., 2007). Previous studies have revealed a deleterious effect of t-PA following TBI; t-PA−/− mice subjected to TBI displayed reduced oedema within 24 h post-TBI and reduced cortical lesion volume at 7 days post TBI (Mori et al., 2001) while intravenous administration of human t-PA to pigs subjected to a fluid percussion injury model of TBI increased brain water content (Armstead et al., 2006) through a mechanism that involves increased vasodilatation and mitogen activated protein kinase activation (Armstead et al., 2011).

In this article, we have demonstrated that measuring extravasated albumin in PBS-perfused brain tissue is an accurate method of studying changes in neurovascular permeability. The ELISA method requires tissue lysates to be prepared by regular methods such as homogenizing in PBS with Triton-TM X-100 and does not require precipitation of proteins. Hence these homogenates can be used for other proteomic analyses such as ELISAs, immunoblotting.
and activity assays. Thus, it enables a reduction of the number of experimental animals, as multiple readouts are obtained from a single cohort of mice.

Here, we show that the intracortical injection of the t-PA:PAI1 complex in t-PA−/− mice after TBI recapitulates the extent of albumin extravasation and MMP3 induction observed in injured wild-type mice. Importantly, the degree of neurovascular permeability correlates with a poorer neurological outcome as early as 3 h post-injury, as measured by the ventral plane video imaging apparatus (DigiGait™). We also show that injection of the t-PA:PAI1 complex directly into the CSF in uninjured mice triggers disruption of cerebrovascular integrity leading to extravasation of albumin into the brain parenchyma within 1 h. We speculate that this effect of t-PA:PAI1 complex formation may be a physiological response during injury (that is overwhelmed during TBI) possibly to increase energy availability.

Our data parallel a report by Yepes et al. (2003) showing an increase in neurovascular breakdown following intraventricular injection of active t-PA in wild-type. Active t-PA, through interaction with LDLR-related protein, directly caused neurovascular breakdown.

**Figure 5** t-PA:PAI1 complex and MMP3 are elevated after injury in CSF of trauma patients, and concentrations of MMP3 and albumin are increased in the injured cortex of human TBI cases. (A(i)) t-PA:PAI1 complex concentration is elevated in patients with GOS-E score 1–2 (death, vegetative state; n = 7), low in patients with GOS-E score 3–4 (severe disability; n = 10) and undetectable in patients with GOS-E score of 5–8 (moderate disability to good recovery; n = 6) and in all control patients (n = 8); t-PA:PAI1 complex levels correlate with neurological outcome (r = −0.539; P = 0.0079, see Supplementary Fig. 1A). (A(ii)) MMP3 concentration in CSF also increases with injury severity, and correlates with the t-PA:PAI1 complex (r = 0.585; P = 0.0006, Supplementary Fig. 1B). (B) Gelatin zymogram shows marked elevation in MMP9 and likely MMP9:TIMP-1 complexes but not MMP2 activity in CSF of 12 patients with TBI (GOS-E score 1–4; indicated below gel). MMP9 is absent or weakly detected in eight non-TBI control patients. MMP2/9 positive control is conditioned media of HT-1080 fibrosarcoma cells. The results presented comprise images from four separate (non-contiguous) zymograms of CSF samples from control and patients with TBI; control and TBI sample lanes have been grouped for clarity. The individual gels are separated by a white line. (C(i)) Increased albumin content in brain tissue of patients with TBI compared with non-TBI control cases (n = 10) within 1 h of injury (n = 6) and a further increased in patients who survived ≥6 h post-injury (n = 6) (Table 1). (ii) MMP3 antigen levels in the same cortical tissue are significantly elevated at ≥6 h post-injury compared with non-TBI control patients. Data expressed as mean ± SD.
dysfunction, independently of plasminogen and MMP9. It was speculated that t-PA associated with LDLR-related protein to cause neurovascular damage through a specific cell signalling event. It remains to be seen if active t-PA also acts on the neurovascular unit following TBI [i.e. through its proteolytic action as seen in models of ischaemic stroke (Su et al., 2008)].

The source of PAI1 could be neuronal or astrocytic or could be derived from the blood following TBI, although the concentration of the t-PA:PAI1 complex in serum derived from a subset of the patients with TBI did not correlate with the concentration in the corresponding CSF sample (Supplementary Fig. 1D). Similarly, t-PA is likely to be derived from the CNS given the high expression of this protease in this compartment (Sashindranath et al., 2011) compared with the circulation. We also note that t-PA:PAI1 complex levels are reported to be higher in serum than in the corresponding plasma (Nilsson et al., 2005) due to the clotting process, hence future studies should use plasma samples to provide a better baseline value. Nonetheless, even though the source of the t-PA and PAI1 observed in the CSF of these patients with TBI remains to be formally determined, our findings suggest that regardless of its source, the increased affinity of the t-PA:PAI1 complex for LDLRs could trigger signalling cascades leading to induction of MMP3, a protease that can actively degrade the basal lamina of the neurovascular unit.

An emerging consensus regarding t-PA-mediated disruption of neurovascular integrity is that it: (i) requires proteolytic activity; (ii) is independent of plasmin; and (iii) involves engagement with an LDLR family member. Our data showing the deleterious effects of the t-PA:PAI1 complex on the neurovasculature provide a novel paradigm that links these events in TBI. Furthermore, since the binding affinity of t-PA for LDLRs is amplified over 300-fold upon complex formation (Nykjaer et al., 1994), we suggest that PAI1 serves as a conduit for t-PA in LDLR signalling and consequent abrogation of neurovascular integrity. It remains to be determined if complex formation between t-PA and other serpins (i.e. neuroserpin and protease nexin-1) also produces similar effects after trauma or if this is a unique property of the t-PA:PAI1 complex.

Since t-PA mediated proteolysis has independently been associated with promotion of neurovascular permeability in other CNS paradigms (Polavarapu et al., 2007; Su et al., 2008), it would have been reasonably assumed that abrogation of t-PA activity by its cognate inhibitors and subsequent internalization of complexes would reverse these deleterious effects. Indeed, intravenous delivery of t-PA into uninjured mice was reported to increase neurovascular permeability in a process that was inhibited by intravenous co-treatment with PAI1 (Fanne et al., 2010). However, our findings indicate that the opposite can actually occur, since injection of PAI1 into the brain parenchyma promotes neurovascular permeability by the formation of the t-PA:PAI1 complex.

The MMPs comprise a second major proteolytic system in the mammalian brain and are also pivotal to CNS function, being involved in tissue remodelling during development and angiogenesis. However, a balance between production, activation and inhibition of MMPs must be maintained to prevent excessive proteolysis or inhibition (Gary, 2002). MMP3 and MMP9 in particular are rapidly induced after CNS injury and mediate neurovascular breakdown in vivo by degrading the basal lamina and tight junction proteins (Gurney et al., 2006). Expression and/or immunoreactivity of MMP3, predominantly in astrocytes (Falo et al., 2006), is increased in other models of TBI (Kim et al., 2005). Here, we show that MMP3 is rapidly induced after controlled cortical impact-induced TBI in mice, as well as in human brain tissue and CSF after trauma. We demonstrate that the t-PA:PAI1 complex singularly promotes an increase in MMP3 concentrations and a correlative increase in albumin extravasation after TBI. Remarkably, levels of t-PA:PAI1 complex correlate with that of MMP3 in CSF of human trauma cases (Supplementary Fig. 1B) further suggesting that the rapid induction of MMP3 after TBI is consequent to t-PA:PAI1 complex formation. MMP3 is likely to facilitate disruption of the cerebrovasculature in trauma as pharmacological inhibition of MMP3 remarkably reduces the extent of albumin extravasation and improves neurological outcome in the acute phase (i.e. within 3 h of injury). These novel data place MMP3 as an important mediator of proteolytic degradation of the neurovascular unit in acute brain injury. MMP3 is also an activator of MMP9 (Candelario-Jallil et al., 2009); however, since inhibition of MMP9 did not protect the neurovascular unit after TBI (not shown), it is unlikely that MMP9 is downstream of MMP3 induction and t-PA:PAI1 complex signalling.

Accumulating data suggest that increased cerebrovascular permeability is responsible for short and long term brain damage after TBI (Shlosberg et al., 2010). Yet, unlike other neurodegenerative diseases, very little is known about the molecular mechanisms that lead to altered neurovascular integrity in neurotrauma. Our study demonstrates that t-PA:PAI1 complex alone is sufficient to disrupt neurovascular integrity by inducing MMP3 after TBI, and is the first thorough description of a link between the plasminogen activator and MMP systems in TBI. Although t-PA:PAI1 complex formation has not been associated with neurovascular disruption in ischaemic stroke thus far, the findings from our TBI model suggest that similar mechanisms occur. Indeed, high levels of t-PA:PAI1 complex in human plasma are reported to be a risk factor in stroke (Johansson et al., 2000). Levels of t-PA:PAI1 complex, as well as immunoreactivity for MMP3 and LDLRs are also increased in the brains of patients with chronic multiple sclerosis (Maeda and Sobel, 1996; Gveric et al., 2003, 2005) suggesting that t-PA:PAI1 complex formation may be associated with altered neurovascular permeability in a broader context. We propose that the t-PA:PAI1 complex and MMP3 are highly relevant targets for therapeutic intervention in TBI and other CNS diseases where neurovascular integrity is compromised.

Acknowledgements

The authors thank Dr Mark Habgood and Prof Norman Saunders for guidance in establishing the controlled cortical impact apparatus; Mr Darren Karadimos and Ms Jessica Farrugia for assistance in experiments; Mr Be’eri Niego for helpful discussions and Dr Anita Horvath for assistance with biochemical techniques.
Funding

Grant numbers 606660 and D0-34 awarded to R.L.M from the National Health and Medical Research Council (NHMRC) of Australia and the Victorian Neurotrauma Initiative, respectively, and by grant numbers HL55374, HL54710, and HL089407 awarded to D.A.L from the National Institutes of Health. Tissues were received from the Neurotrauma Tissue/Fluid Bank, supported by the Victorian Brain Bank Network, Mental Health Research Institute, The Alfred Hospital, the National Trauma Research Institute, Victorian Forensic Institute of Medicine and funded by Victorian Neurotrauma Initiative, the Transport Accident Commission (TAC) and the NHMRC of Australia.

Supplementary material

Supplementary material is available at Brain online.

References

Armstead WM, Kiessling JW, Riley J, Cines DB, Higazi AA. tPA contributes to impaired NMDA cerebrovasodilation after traumatic brain injury through activation of JNK MAPK. Neurol Res 2011; 33: 726–33.


