Contributions of multiple proteases to neurotoxicity in a mouse model of intracerebral haemorrhage

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Proteases such as matrix metalloproteinases (MMPs) and thrombin are implicated in intracerebral haemorrhage (ICH) but their interactions amongst one another and interdependency remain to be defined. The latter is important since proteases acting through different mechanisms to inflict neurotoxicity would require separate targeting compared with proteases acting through the same cascade. We reported recently that MMP-9 and thrombin combined to promote neurotoxicity in ICH; however, as there was still substantial injury when both MMP-9 and thrombin were inhibited, we sought other factors that also contribute to ICH pathology. MMP-3, another member of the MMP family, has been correlated with poor prognosis in ICH in humans and it has been shown to increase rapidly after ICH in animals. Moreover, MMP-3 can convert the MMP-9 zymogen to its active form. Thus, we have examined whether MMP-3 is neurotoxic and addressed whether its potential effect in ICH is dependent on, or additional to, damage inflicted by MMP-9 and thrombin. We report that cultured neurons are killed by MMP-3 and that neuronal death is most marked when all three proteases, MMP-3, MMP-9 and thrombin, are combined. In vivo, the injection of autologous blood into the right striatum to produce ICH injury resulted in MMP-3 expression within 3 h. The blood-induced lesion and neuronal death was significantly reduced in MMP-3 or MMP-9 null mice compared with wild-type counterparts, and MMP-3 and -9 double null mice had even less brain damage. Significantly, pathological destruction after ICH was least in MMP-3 and -9 double null mice treated with a thrombin antagonist, hirudin. These results provide insights into molecules that inflict neurotoxicity in ICH and demonstrate that multiple proteases would need to be targeted simultaneously to successfully reduce ICH neurotoxicity.

Keywords: metalloproteinases; MMPs; neuronal death; stroke; thrombin

Abbreviations: ATP = adenosine triphosphate; ECM = extracellular matrix; FJ = Fluoro-Jade; HFNs = human foetal neurons; ICH = intracerebral haemorrhage; MAP-2 = microtubule-associated protein-2; MMP = matrix metalloproteinase; NeuN = neuronal nuclear protein; PBS = phosphate buffered saline; PFA = paraformadehyde

Introduction

Intracerebral haemorrhage (ICH) is the result of the rupture of cerebral vessels leading to the development of a haematoma in the brain parenchyma. While ICH accounts for a small proportion (10–15%) of all strokes, it exerts a worse prognosis than injury caused by the predominant form of stroke, ischaemia. The main predictor of outcome with ICH is haematoma volume within the
parenchyma and ventricles. Aside from the space-occupying hematoma that distends neural structures, the presence of blood in ICH releases detrimental factors into the brain microenvironment, including inflammatory molecules, the pro-oxidants haeme and ferrous ion and proteolytic enzymes such as matrix metalloproteinases (MMPs) and thrombin (Wang and Tsirka, 2005; Xue et al., 2006; Hua et al., 2007; Wang and Dore, 2007). Effective medical treatment for ICH has been unsatisfactory (Broderick et al., 2007) and the prognosis of ICH remains poor.

In previous work, we reported that thrombin and MMP-9 help account for neurotoxicity of ICH within the first 24 h of injury (Xue et al., 2006). Nonetheless, substantial amount of injury to the brain parenchyma persisted even when both thrombin and MMP-9 were inhibited (Xue et al., 2006), suggesting that other mediators contributed to the brain injury. In particular, it is possible that other proteases interact with MMP-9 and thrombin to mediate neurotoxicity.

Considering other proteases in ICH, MMP-3 deserves attention. MMP-3 is one of the first MMPs to be elevated following ICH injury in mice (Rosenberg et al., 2001; Wells et al., 2005; Grossetete and Rosenberg, 2008). In a related model where tissue-type plasminogen activator (tPA) is used to induce intracerebral bleed after ischaemia, the degree of haemorrhage is less in MMP-3 null mice relative to wild-type controls (Suzuki et al., 2007). In clinical studies, MMP-3 elevation is notable in the plasma of patients with ICH injury and increased MMP-3 is correlated with higher risk of mortality (Alvarez-Sabin et al., 2006). In some experiments, the broad-spectrum MMP inhibitors BB94 (British Biotech, UK, 500 nM) and GM6001 (Calbiochem, La Jolla, 5 μg/ml) were applied to cultures 30 min before the addition of MMP-3.

### Evaluations of neuronal death in culture

Forty-eight hours after treatment with proteases, neurons were fixed with 4% paraformaldehyde (PFA), washed with phosphate buffered saline (PBS) and then incubated with mouse anti-human microtubule-associated protein-2 (MAP-2) antibody (Sigma, 1:2000) for 1 h to label neurons. Active caspase-3 polyclonal antibody (New England Biolabs, Ipswich, MA, 1:200) was used to identify apoptotic cells. A goat anti-mouse Ig conjugated to Cy3 (Caltag Laboratories, Burlingame, CA, 1:300) and anti rabbit Alexa 488 (Invitrogen, Eugene, OR, 1:400) were used as secondary antibodies for another hour. Hoechst dye (Sigma, nuclear yellow, 1:100) was applied for 10 min to label all nuclei and slides were covered with gelvatol (Vecil et al., 2000). Using an immunofluorescence microscope, four pre-designated fields of each well at 400× magnification were evaluated for the number of remaining neurons for that well. The neuronal numbers remaining in the four defined fields per well are expressed as per cent of that in control cultures.

### Assessment of neuronal viability in culture by ATP luminescence assays

About 100 000 HFN per well were plated in opaque-walled 96-multwell plates with 100 μl culture medium. The control wells contained medium without cells to obtain a value for background luminescence. MMP-3,-9 and thrombin or in combination were added to the cultured HFN wells and incubated for 48 h. The wells were equilibrated to room temperature for ~30 min and 100 μl of CellTiter-Glo® Reagent (Promega Corporation, Madison, WI) was then added to the cell culture medium. The contents were mixed for 5 min on an orbital shaker to induce cell lysis. The plates were then incubated at room temperature for 10 min to stabilize luminescent signal. Luminescence was recorded by using a luminescence scanner (Luminoskan Ascent, Germany).

### Mice, ICH injury and treatment

MMP-3,-9 single and MMP-3/-9 double null mice as well as background matched wild-type (129/SvEv) 7- to 8-weeks-old mice were used for this study. The MMP-9 null mice were originally obtained from Dr Zena Werb (University of California, San Francisco) (Vu et al., 1998) and are bred in-house. The MMP-3 null mice were originally generated by Mudgett et al. (1998) and were generously provided by Dr Lynn Matrisian on the 129/SvEv background.

### Material and Methods

#### Culture of human foetal neurons (HFN) and their treatment

Neurons were obtained from the brains of specimens of 15–22 weeks gestation; these specimens were from therapeutic abortions and their use for research has been approved by local institutional human ethics committee. We have detailed the methods for obtaining HFN and the reader is referred elsewhere for the technique (Vecil et al., 2000). For toxicity experiments involving immunofluorescence analyses, cells at density of 100 000 cells/well were contained within 16-well Lab-tek slides (Nunc, Naperville, IL). For toxicity experiments that employed adenosine triphosphate (ATP) luminescence assays, cells were seeded at 100 000 cells/well of 96-well plates. Active catalytic domain recombinant human MMP-3 (22 kDa, Calbiochem, Catalog # 444217, La Jolla, CA) was used at final concentrations of 50–200 ng/ml and active recombinant human MMP-9 (83 kDa, Calbiochem, Catalog # PF024, La Jolla, CA) was tested at 100 ng/ml. Thrombin (human plasma, T4393, Sigma, St Louis, MO) was used at a final concentration of 2 U/ml. These concentrations of MMP-9 and thrombin were previously reported by us to be neurotoxic (Xue et al., 2006). In some experiments, the broad-spectrum MMP inhibitors BB94 (British Biotech, UK, 500 nM) and GM6001 (Calbiochem, La Jolla, 5 μg/ml) were applied to cultures 30 min before the addition of MMP-3.
Genotyping: DNA preparation and PCR

The genotype of mice was determined by PCR analysis of DNA that was extracted from a 2 mm diameter of the ear obtained through an ear punch. The ear was digested in 50 μl buffer (50 mM potassium chloride, 10 mM Tris–HCl (pH 8.3), 2.5 mM magnesium chloride, 0.1 mg/ml gelatin, 0.45% nonidet p40, 0.45% tween20, 0.5 mg/ml protein K overnight at 56°C), then 10 min at 95°C for inactivation of proteinase K, the supernatant was taken after centrifuging 5 min at 13,000 r.p.m., 1 μl was used for PCR analysis. The reaction mixture contained 1 x PCR reaction buffer, 200 μM dNTP, 2 mM MgCl₂, 1 μM of each Primer and 0.05 U/μl Taq polymerase (Invitrogen) in a total volume of 50 μl. The primers and band size were: wild-type for MMP-9 (5'-CTC GCG GCA AGT CTG CAT A-3', 5'-GTG GCA TCA TAA CAT CAC A-3'), band of 277 bp; MMP-9 null (5'-CTC GCG GCA AGT CTG CAT A-3', 5'-GTG GCA TCA TAA CAT CAC A-3'), band of 500 bp; wild-type for MMP3: (5'-GGC TTC TGC CAT TGA AAA AG-3', 5'-CTG GTT ATG AGG ACA TCC C-3'), band of 410 bp and MMP-3 null (5'-CAA GAC AGA GTG TGG ATTTC-3', 5'-CCG ACC GCT ATC AGG ACA T-3'), band of 700 bp. The reaction consisted of 50 cycles: denaturation at 95°C for 30 s; annealing at 62°C for 30 s; elongation at 72°C for 30 s. The PCR products were analysed by using Tris–ACETATE-EDTA buffer agarose electrophoresis (1.2%) labelled with ethidium bromide. The bands were visualized by using a Syngene GBOX (Frederick, MD, USA) and images were taken by LAS-100 cooled CCD camera (Fujifilm, Tokyo, Japan).

Histopathological evaluation

Mice were killed at 24 h after ICH and were fixed with 10% formalin through the heart perfusion. The brains were fixed in the same fixative for 1–5 days. Fixed brains were dehydrated and embedded in paraffin. Consecutive sections (6 μm) from three levels 250 μm apart (Fig. 4) were collected. Haematoxylin and eosin (H&E) staining was used to assess brain damage area. The damaged area of each section was traced and tabulated by using an imagePro software. Areas of damaged brain were identified by the presence of blood, tissue rarefaction or necrosis. Fluoro-Jade (FJ) staining was used to assess dying neurons (Xue et al., 2006); at 400×, FJ positive dying neurons were counted in four fields close to the injured areas as previously described (Xue and Del Bigio, 2005). The values from three sections analysed per mouse were pooled to obtain the area of brain damage or the number of FJ dying neurons per mouse.

Cryostat sections were used to investigate MMP-3 expression that occurred after ICH, since the relevant antigen could not be detected reliably on paraffin sections. Wild-type mice were killed at 3, 6 and 24 h (3–4 mice were used for each time point) after blood injection into brain, perfused through the heart with 5 ml ice cold PBS, following by 10 ml ice cold 4% PFA. Frozen brain sections (15 μm) were cut serially through the damaged brain by cryostat, washed with PBS and incubated with primary rat anti mouse MMP-3 antibody (diluted 1/100 in HHG, R&D Canada) at 4°C overnight. Slides were then washed, incubated in goat anti rat Cy3 (1/300) at 4°C for 1 h, and further labelled for neurons by using neuronal nuclear protein (NeuN) conjugated Alex 488 (1 : 300). After labelling with Hoescht dye (Sigma, nuclear yellow, 1 : 100), slides were mounted with gelvatol (Vecil et al., 2000). An Olympus Fluoview confocal microscope (Olympus Canada Inc. Markham, ON) was used to observe the double staining and to take the images.

Statistical analysis

All data are expressed as mean±SD. Intergroup comparisons were made by ANOVA followed by Bonferroni. The differences were considered significantly when P<0.05. We used GraphPad InStat software for statistical analyses.

Results

MMP-3 kills neurons in culture and it increases the toxicity of thrombin and/or MMP-9

We first tested the potential toxicity of proteases using cells in culture. HFNs were chosen as the test bed since recombinant MMP-3 and -9 were available in the human form. Figure 1A and B presents micrographs of cultures and emphasizes the high purity of the human neurons since the majority of Hoechst-positive nuclei corresponds with microtubule associated protein (MAP)-2 positive neurons. Application of MMP-3 to HFN reduced the number of neurons, a manifestation of toxicity and this was apparent by 24 h which became marked at 48 h (Fig. 1C).

To further support the neurotoxic potential of MMP-3, we determined the induction of active caspase-3 which signifies a cell committed to apoptosis. While we did not observe cells that were positive for active caspase-3 in control conditions (Fig. 1D–G), MMP-3 treated neurons had expression of active caspase-3 (Fig. 1H–K; arrow in I and K).

To quantitate neurotoxicity, we counted the number of MAP-2 positive neurons that remained after 48 h of treatment. Compared to controls, cultures treated with 100 and 200 ng/ml active MMP-3 had fewer cells (Supplementary Fig. 1A) and this manifestation of MMP-3 toxicity was corroborated by using the ATP luminescence assay (Supplementary Fig. 1B). In this regard, the correlation coefficient (determined by regression analysis) between MAP-2 counts and ATP levels in control and MMP-3 treated cultures was 0.65.
$r^2, P < 0.001$, indicating that there was good correspondence of MMP-3 mediated cell death as determined by two methods.

We addressed whether the toxicity of MMP-3 was related to its protease activity or through non-proteolytic functions of the enzyme. Cultured HFNs were pretreated for 30 min with one of two inhibitors of MMP protease activity, namely GM6001 and BB94 (Wang and Tsirka, 2005; Hu et al., 2007) and active MMP-3 was then added. We found that the toxicity of MMP-3 on HFN was prevented by both GM6001 and BB94 (Supplementary Fig. 1C).

We next examined whether there was combinational effect of MMP-3 neurotoxicity with thrombin and/or MMP-9 in vitro. Figure 2A shows that the combination of MMP-3 with MMP-9 or thrombin resulted in a significantly greater death of neurons compared with the proteases used alone. Neuronal loss was the most pronounced when the combination of all three agents was used, assessed using MAP-2 staining (Fig. 2A) and ATP luminescence assay (Fig. 2B).

In summary, the tissue culture experiments show that MMP-3, -9 and thrombin kill neurons singly, and that their combination increases neurotoxicity. We sought then to investigate the potential of these proteases to be neurotoxic in vivo in the context of acute neurotoxicity in ICH.

**ICH leads to local MMP-3 expression which promotes neurotoxicity**

In correspondence with our previous report (Xue et al., 2006), an injection of $10 \mu l$ of saline into the striatum produced at 24 h minimal injury to that area (Fig. 3A). In contrast, $10 \mu l$ of blood injected into the striatum resulted in an extensive area of striatal injury that includes oedema and necrosis (Fig. 3B).

The regional and cellular localization of MMP-3 in the brain of wild-type mice subjected to ICH was studied by...
immunohistochemistry. In non-injured control striatum, few cells were MMP-3 positive (Fig. 3C). In striatum of mice injected with saline, a few more cells over the non-injured condition were MMP-3 positive (data not shown). In contrast, by 3 h of ICH, there were numerous MMP-3 positive cells detected (Fig. 3D) and these were confined to the area of injury; staining pattern at 24 h resembled that at 3 h (data not shown). Using a NeuN antibody to detect neurons, we find that a significant number of MMP-3 positive cells colocalized to neurons (Fig. 3E and F). Thus, MMP-3 expression was upregulated promptly following ICH, placing this protease in the vicinity of injury to potentially inflict damage in vivo.

To determine whether local MMP-3 expression could indeed produce injury, we injected autologous blood into the striatum of wild-type mice and assessed the histopathology in a blinded manner. We focussed on the acute injury by 24 h of ICH since significant loss of neurons already occurred at this time period (Xue et al., 2006). Using sections from three specified levels of the brain (Fig. 4A) spaced 250 μm apart and beginning consecutively from the epicentre of injury, we traced the area of brain damage within each section as previously described (Xue et al., 2006). In this manner, we determined that the injection of autologous blood into wild-type brain resulted in significant brain damage (Fig. 4B and C). In contrast, the same volume of autologous blood into the brain of MMP-3 null mice resulted in less area of brain damage (Fig. 4B and C) compared with that in wild-type. Similarly, the quantitative counts of FJ positive cells, a marker of dying neurons (Fig. 4B–D), demonstrates that neuronal death following ICH is significantly reduced in MMP-3 null mice compared with wild-type animals.

MMP-3 and -9 produce additive and independent neurotoxic effects on the brain parenchyma

The above experiments implicate MMP-3 expressed following ICH as being neurotoxic since MMP-3 null mice without this
protease have less cerebral injury compared with wild-type controls. Previous experiments (Xue et al., 2006) have determined that MMP-9 null mice are also partially protected from ICH. Thus, both MMP-3 and MMP-9 are involved in ICH and it remains to be determined whether they act linearly and are interdependent on each other or whether they exert toxicity exclusive of one another. To address this, we first injected either activated MMP-3 or -9 into the brains of wild-type animals to document

Fig. 4  ICH is less damaging in MMP-3 null mice compared with wild-type controls. The manifestation of brain injury is evaluated at three levels spaced 250 μm apart from the epicentre of injury (lines in A). Representative HE stain shows that 10 μl of blood produces extensive brain damage in the right striatum of wild-type mice compared with that in MMP-3 null mice (B), and this is verified by quantitation of the area of brain damage within each section, and combining the areas from all three sections of individual mice (C). FJ staining (B) shows increased numbers of dying neurons in wild-type compared with that in MMP-3 null brain (D) in four areas adjacent to the haematoma following ICH. In C and D, each individual ellipse represents data from a single mouse while the black solid square depicts the mean of data from mice in each group. "P < 0.05, ""P < 0.01 compared with wild-type mice injected with blood.
their intrinsic neurotoxic potential. Following an initial dosing of 0.1 μg protease in 5 μl volume which did not produce additional damage compared with vehicle (data not shown), we focused on a higher 0.5 μg/5 μl dose; others have injected 1 μg of active MMP-9 into the cortical white matter of rats and reported axonal injury (Newman et al., 2001). Twenty-four hours following the injection of active MMP-3 or -9 into the striatum of wild-type mice, an increase in the area of brain damage and in the number of dying neurons was found compared with vehicle injection (Supplementary Fig. 2A and B). The extent of toxicity produced by both proteases was equivalent. Active MMP-9 was then injected into MMP-3 null brains and the converse was performed. If these proteases were dependent on each other’s activity, then the outcome of the injection of the individual protease in the null mice should not differ from that of the sham vehicle injection group. Supplementary Figure 2 shows that the level of toxicity produced by active MMP-3 or -9 in the respective MMP-9 or -3 null mice is higher than that elicited by vehicle injection, indicating that these proteases are not dependent on one another’s presence in mediating neurotoxicity.

The results of MMP-3 and -9 acting independently of one another imply that both MMP-3 and -9 exert their own toxic consequences in vivo. To test this further, we first generated MMP-3 and -9 double null mice, following which autologous blood was injected into the striatum of wild-type or MMP-3 and -9 single null, or MMP-3/-9 double null mice. Significantly, while there was reduced brain damage and dying neurons in MMP-3 or -9 single null mice compared with wild-type animals, the least extent of brain damage and dying neurons occurred in the double null mice (Fig. 5). These results emphasize that the mode of toxicity in ICH involves MMP-3 and -9, that these proteases are not reliant on one another in ICH, and that their individual activities combine for additive neurotoxicity in vivo.

Concurrent neurotoxic effects of MMP-3, MMP-9 and thrombin in ICH injury

Thrombin is implicated in mediating neuronal death in ICH (Xue and Del Bigio, 2005; Hua et al., 2007) and in interacting with MMP-9 (Xue et al., 2006), even while its coagulant effect is desirable for stemming bleeding and reducing the size of the haematoma after an intracranial bleed (Mayer et al., 2005). For the current work, we investigated the possible collaboration of MMP-3 and thrombin in ICH. To probe the role of thrombin, we administered the thrombin antagonist, hirudin (Fenton et al., 1991), within the autologous blood that was used to inflict ICH in the striatum. Figure 6 shows a smaller area of brain damage (Fig. 6) and lower number of dying neurons following ICH in wild-type mice treated with hirudin compared with vehicle, providing support for the toxicity of thrombin. Notably, an even smaller extent of brain injury occurred in mice with both MMP-3 deficiency and hirudin treatment, compared with all other groups, indicating that the mode of toxicity in ICH involves the additive effect of thrombin and MMP-3.

Discussion

ICH carries a dismal prognosis due to brain ischaemia, mechanical disruption of brain tissue caused by the enlarging haematoma, oedema and rise in intracranial pressure and blood-induced brain injury. The toxicity of blood occurs through a number of mechanisms including the direct lytic effect of blood components and the recruitment into the brain parenchyma of inflammatory cells. The latter release a spectrum of injurious products including proteolytic enzymes, which are also contributed by neural cells upon injury. A group of proteolytic enzymes associated with ICH is the MMPs (Wang and Tsirka, 2005; Xue and Del Bigio, 2005; Xue et al., 2006; Hua et al., 2007; Wang and Dore, 2007). In this regard, numerous reports document the increase in MMP-9 either in the systemic circulation or brain of patients with ICH (Abilleira et al., 2003; Castellanos et al., 2003; Rosell et al., 2006), and in
animal models of the condition (Rosenberg and Navratil, 1997; Power et al., 2003; Wang and Tsirka, 2005). Another protease that is implicated in ICH is thrombin, which can be toxic in high concentrations even though its activity is advantageous for blood clotting to stem the enlarging haematoma (Xue and Del Bigio, 2005; Hua et al., 2007).

The increase of MMP-9 and thrombin after ICH suggests their potential for interactions. Indeed, we observed that while both thrombin and MMP-9 could act independently to promote injury after ICH in mice, thrombin also exerted its neurotoxicity partially through the activation of MMP-9 (Xue et al., 2006). When we inhibited both MMP-9 and thrombin after ICH and reduced the extent of damage to the mouse brain parenchyma at 24 h compared to untreated ICH controls, there was still significant resultant injury (Xue et al., 2006) indicating that other factors also mediate neurotoxicity following ICH.

With regards to additional factors, we considered the potential role of MMP-12 in producing ICH injury, since MMP-12 null mice recover better functional indices 14 days after ICH compared with wild-type mice (Wells et al., 2005); however, the rise in MMP-12 levels tends to be substantial only days after the insult (Power et al., 2003; Wells et al., 2005). An MMP member that deserves particular attention for acute neurotoxicity after ICH is MMP-3, since this protease elevates within 24 h after ICH in mice (Wells et al., 2005) and rats (Power et al., 2003; Grossetete and Rosenberg, 2008). Moreover, after ICH in humans, blood plasma levels of MMP-3 (and -9) are positively correlated with the residual scar volume at 3 months (Abilleira et al., 2003; Alvarez-Sabin et al., 2004). The extent of elevation of MMP-3 in plasma is correlated with the probability of mortality after ICH in humans (Alvarez-Sabin et al., 2004). More recently, the intracranial bleeding after tPA treatment of stroke in mice was found to be ameliorated in MMP-3 null, but not MMP-9 null, mice compared to wild-type controls, implicating MMP-3 in ITPA neurotoxicity (Suzuki et al., 2007).

Given these discussions, we have assessed the potential contribution of MMP-3 to neurotoxicity after ICH. We find MMP-3 to...
play a role in mediating brain damage since MMP-3 kills neurons when applied in culture (Supplementary Fig. 1) and in vivo (Fig. 4), and given that MMP-3 null mice have less ICH-induced brain damage and neuronal death compared to wild-type controls (Fig. 5). Importantly, MMP-3 adds to the toxicity of MMP-9 and of thrombin, and this is evident in culture (Fig. 2) and after ICH (Figs 5–7). When all three proteases are inhibited by using the thrombin antagonist hirudin in MMP-3 and -9 double null mice, the extent of neurotoxicity is least (Fig. 7). Collectively, these results implicate the concurrent injury induced by all three proteases in the early periods after ICH.

A deficit of this work is that while we measured lesion size as determined by the perimeter of lesions, we did not address whether proteases contribute to tissue cavitation. A cavity was observed at 24 h after ICH in many mice, but we did not measure the size of the centre cavity. Future studies will address this and will also consider whether the cavitation impacts long-term outcomes.

We consider it likely that MMP-3, -9 and thrombin could also interact with other molecules that accumulate after ICH. A recent manuscript described that a haemoglobin derivative, ß-haematin, assisted in the MMP-3-mediated activation of MMP-9 (Geurts et al., 2008). Thus, complex chemical reactions could be unleashed following ICH. We note that we did perform experiments to address whether MMP-3 levels could be altered in MMP-9 null mice due to compensation or whether MMP-9 levels could be altered in MMP-3 null mice. Our data indicate that at 24 h following ICH, MMP-3 levels in the striatum of WT mice did not differ from those of MMP-9 null mice (Supplementary Fig. 3). Similarly, MMP-9 levels in the striatum of WT mice did not differ from those of MMP-3 null mice (92% of wild-type) after ICH.

While several reports demonstrate the toxicity of MMP-9 and thrombin on neurons (Yong et al., 2001; Gu et al., 2002; Jourquin et al., 2003; Hua et al., 2007), we were unable to find reports in the literature of the direct toxicity of MMP-3 on neurons. An indirect neurotoxic mechanism for MMP-3 has been described, in that MMP-3 activates microglia that then releases neurotoxic products (Kim et al., 2005). Continued MMP-3 expression has been correlated with maladaptive synaptic plasticity caused by brain injury in contrast to adaptive synaptic plasticity, and this inferred rather than proven direct MMP-3 neurotoxicity (Falo et al., 2006). When injected into the brain, outcomes of MMP-3 have included increased BBB permeability and the facilitation of neutrophil influx (Gurney et al., 2006). Overall, our demonstration that MMP-3 kills human neurons in cultures that are virtually devoid of microglia emphasizes that neurons can become direct targets of MMP-3 toxicity.

In the current study, we have found MMP-3 to be elevated by 3 h in the brain parenchyma following ICH (Fig. 3). By double immunoflourescence analysis with NeuN, we find that many of the MMP-3 expressing cells are neurons. Others have reported that in culture, MMP-3 is released from apoptotic neuronal cells and that this then activates microglia, which further exacerbates neuronal degeneration by generation of cytokines (Kim et al., 2005); a similar mechanism of neuron-derived MMP-3 activating microglia to produce toxicity to dopaminergic neurons in vivo has also been described (Kim et al., 2007). In addition to neurons, we note that microglia (Rosenberg et al., 2001), reactive astrocytes (Falo et al., 2006) and endothelial cells (Suzuki et al., 2007) can also be sources of MMP-3 after neural injury in vivo.

How may MMPs including MMP-3 kill neurons? Several mechanisms are likely involved. First, MMPs can degrade all components of the extracellular matrix (ECM); as ECM-integrin signalling is important in the regulation of cell survival (Yong et al., 2001; Yong, 2005; Page-McCaw et al., 2007). In other paradigms, neuronal death in the hippocampus after kainate-induced seizures has been attributed to the degradation of proteases by laminin (Chen and Strickland, 1997). Second, MMPs can directly alter receptors associated with death signalling, such as Fasl and tumour necrosis factor-α receptor (Powell et al., 1999). Third, MMPs including MMP-3 has been shown to translocate into the nucleus of cells where the result is apoptosis (Si-Tayeb et al., 2006). The interaction of MMPs with other molecules in the injured CNS is also crucial for determining whether toxicity occurs. For example, nitric oxide interacts with MMP-9 to form S-nitrosylated MMP-9 which is neurotoxic in vitro and in a model of brain ischaemia (Gu et al., 2002).

With regards to whether different or similar mechanisms of neurotoxicity account for the individual or collective actions of MMP-3, -9 or thrombin, this remains to be established. Given that there is additive impact on neurotoxicity, it seems likely that different mechanisms or substrates are operative. However, there could also be some overlapping actions, since MMP-3 or thrombin could both activate MMP-9.

In contrast to toxicity of MMPs, it has been reported that MMP-3 is neuroprotective in some context. Wetzel et al. (2003) described that doxorubicin-induced neuronal apoptosis was attenuated by active MMP-3, likely by removing FasL from neuronal surface thereby protecting against Fas-FasL mediated apoptosis. This observation highlights the complex roles of MMPs in the nervous system. In the normal mature CNS, most MMPs are expressed at low levels where they serve physiological functions such as regulating synaptic plasticity, learning and memory (Agrawal et al., 2008). Indeed, we have described MMPs as ‘the good guys gone bad’ in neurological conditions (Agrawal et al., 2008), when a sudden and massive upregulation of several MMP members occur. The sudden increase of MMPs mediate actions that include neurotoxicity, demyelination and BBB leakage (Yong et al., 2001).

The physiologic roles of MMPs in the mature nervous system imply that there could also be beneficial functions for MMP members that are increased in ICH. Indeed, following the early periods after injury when the balance of the upregulation of several MMP members is pathology, the later and subtle rise of some MMP members appears to have useful outcomes. It is thus notable that Zhao et al. (2006) reported that when an MMP inhibitor (FN-439) was given to rats with focal cerebral ischaemia on day 1, infarct volumes were reduced at day 14; when MMP inhibition was delayed until 7 days, infarct volumes at day 14 were worsened. These results have led to an appreciation that while the early massive increase of several MMP members appears detrimental on balance, the later effects of MMPs in remodelling of the injured environment should be spared (Zhao et al., 2006).
The recognition that thrombin can be neurotoxic in ICH complicates the treatment of this condition. Thrombin is essential for the formation of a blood clot and its generation is partially the basis of the utility of activated Factor VIIa in ICH (Mayer et al., 2005). These results emphasize the need to dissect the mechanisms of thrombin neurotoxicity, such that the undesirable mechanisms could be inhibited while sparing the therapeutic blood-clotting functions of thrombin in ICH.

In summary, our results demonstrate that MMP-3 is neurotoxic and that it adds to the neurotoxicity of MMP-9 and thrombin in vitro and during ICH in mice. To improve the prognosis of ICH, the neurotoxic actions of MMP-3, -9 and thrombin must be inhibited early and simultaneously after ICH injury.

**Supplementary material**

Supplementary material is available at *Brain* online.

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