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Matrix metalloproteinase (MMP)-12 expression has a negative impact on sensorimotor function following intracerebral haemorrhage in mice

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Abstract

We investigated the role of matrix metalloproteinases (MMPs) in a mouse model of intracerebral haemorrhage (ICH). Transcripts encoding nine of the 23 known mammalian MMPs were measured. MMP-12 levels were the most elevated. To evaluate the role of MMP-12 in ICH, haemorrhages were induced in wild-type (WT) and MMP-12 null mice. The results show that MMP-12 null mice exhibited significant functional recovery of forelimb reaching and reduced dependence on the ipsilateral forelimb compared to WT mice. There was also a trend for improved sensory function in the tape removal test. With respect to single pellet skilled reaching, MMP-12 null mice recovered to a level that was not significantly different from sham at 14 and 28 days post-ICH. In contrast, WT animals demonstrated a persistent impairment relative to sham controls throughout the survival period ($P < 0.05$). The cylinder task revealed a lesion-induced reliance on the ipsilateral forelimb that was apparent at day 7 in both MMP-12 null and WT mice ($P < 0.05$), but only persisted in WT mice at 14 days post-ICH ($P < 0.05$). Differences in functional outcome could not be explained by tissue sparing. However, Iba1 immunostaining indicated that more cells bearing macrophage morphology were recruited to the lesion area in WT mice. This is the first study to profile the expression patterns of a number of the known MMPs following ICH in mice. The data indicate that MMP-12 expression following haemorrhagic stroke is deleterious and contributes to the development of secondary injury in this disease.

Introduction

Intracerebral haemorrhage (ICH) accounts for approximately 15–20% of all strokes (Qureshi *et al*., 2001). The immediate pathophysiological consequences of ICH include the mechanical compression of the brain parenchyma and cellular elements due to an accumulation of blood and all its components within the tissue and subsequent oedema (Mun-Bryce & Rosenberg, 1998). Blood flow to surrounding tissues is also often impaired producing a penumbral zone akin to focal ischemia (Bullock *et al*., 1988; Hatazawa *et al*., 1995; Obrenovitch, 1995). Subsequently, inflammation ensues with activation of microglia and the influx of neutrophils, followed by macrophages and other leucocytes. Together, these events culminate in axonal damage as well as neuronal and glial cell death.

Recently, a family of proteases known as the matrix metalloproteinases (MMPs) has being implicated in inflammatory processes within the CNS (see Yong *et al*., 2001 for a comprehensive review). MMPs are a family of zinc-dependent endopeptidases capable of degrading all components of the extracellular matrix (ECM). As such they have been shown to be critical in developmental processes as well as wound healing and repair. However, the aberrant expression of several MMPs has been implicated in a number of disease pathologies including cancer metastasis, multiple sclerosis (MS), human immunodeficiency virus (HIV) associated dementia, trauma as well as stroke (Yong *et al*., 2001).

The involvement of MMPs in stroke has largely focused on the role of family member MMP-9 and more often in the context of ischemic stroke or in haemorrhagic transformation after thrombolytic therapy rather than in haemorrhagic stroke alone. In this regard, the up-regulation of MMP-9 has been demonstrated in animal models of ischemia (Rosenberg *et al*., 1996; Fujimura *et al*., 1999; Gasche *et al*., 1999), in human ischemic stroke (Anthony *et al*., 1997; Clark *et al*., 1997) and in cases of haemorrhagic transformation (Montaner *et al*., 2001b; Sumii & Lo, 2002; Castellanos *et al*., 2003; Heo *et al*., 2003; Montaner *et al*., 2003). Furthermore, other studies have demonstrated that the use of a broad-spectrum MMP inhibitor, BB-94, reduces the risk of haemorrhagic transformation after tPA treatment of ischemic stroke (Lapchak *et al*., 2000; Pfefferkorn & Rosenberg, 2003).

With respect to ICH, studies have implicated MMPs as components in the sequelae of adverse events that unfold following ICH. For example, in a rat model of ICH using bacterial collagenase, zymography was used to demonstrate an increase in MMP-9 levels in a time frame that corresponded to oedema formation. Administration of an MMP inhibitor, BB-1101, 6 h after ICH induction resulted in decreased oedema formation at 24 h and thus implicated MMPs in ICH pathology (Rosenberg & Navratil, 1997). In addition, a recent

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study evaluated MMP expression in a rat model of ICH and found elevated levels of RNA transcript for MMPs 2, 3, 7, 9 and most notably MMP-12 (Power *et al.*, 2003). They also reported that minocycline, a tetracycline derivative, was able to significantly improve behavioural outcome in this model and provided evidence that this was correlated with a reduction in ICH-induced MMP-12 expression. Finally, a study of MMPs in patients with acute ICH revealed that plasma MMP-9 levels were significantly increased and that this increase was associated with peri-haematomal oedema and neurological worsening (Abilleira *et al.*, 2003).

The following study attempts to further our understanding of the role of MMPs in ICH. To this end we have profiled the early expression of nine MMPs in a mouse model of ICH. In addition, in order to address the roles of MMP-12 specifically, in particular in ICH, we have used a genetically modified mouse strain lacking MMP-12 protease activity and followed recovery using multiple behavioural tasks.

Methods

Surgical procedures

Mice were anaesthetized using a mixture of halothane (2% induction; 1% maintenance) in 30% O_2 and 70% N_2O. Animals were placed in a stereotaxic frame using modified earbars fitted with blunt rubber ends designed for mice. Rectal temperature was maintained between 36 and 37 °C for the duration of the surgery. Procedures for induction of intracerebral haemorrhage were modified from the procedures designed for rats originally described by Rosenberg and colleagues (Rosenberg *et al.*, 1990b). Following a midline scalp incision, a small burr hole was made and the dura mater was punctured. Using a 30 gauge injector needle, 0.14 U of collagenase (Type IV; Sigma) in 0.3 μL saline was injected over 5 min into the dorsolateral region of the right caudate-putamen (coordinates +0.5 mm anterior, −2.5 mm lateral, −3.5 ventral). The injector needle was left *in situ* for 5 min to minimize backflow. Control animals received an injection of 0.3 μL of saline or no surgical procedures. The scalp was sutured and animals were returned to their home cage, which was warmed with a heated water blanket for approximately 3 h after the surgery.

RNA analysis

Twenty male CD−1 mice (Charles River Laboratories, Montreal, Quebec, Canada) were subjected to intracerebral haemorrhage and killed at 6 h ($n = 4$), 24 h ($n = 4$), or 5 days ($n = 4$) after surgery. They were given an overdose of sodium pentobarbitol, decapitated and brains rapidly removed. Determination of altered MMP expression following ICH was performed by comparison of striatal expression levels in control animals that received either an injection of saline ($n = 4$) or no injection ($n = 4$). Brains were removed and the striata (contralateral and ipsilateral to the injection site) were isolated, placed in small centrifuge tubes and snap frozen in liquid nitrogen for subsequent RNA isolation. Tissue was thawed and homogenized in Trizol reagent and total RNA extracted as previously described (Rostworowski *et al.*, 1997). Using a multiprobe RNase Protection Assay (Pagenstecher *et al.*, 1998), the expression of nine MMP family members was investigated. Briefly, 8 μg of total RNA were used for each sample. Samples were heated to 95 °C for unfolding of RNA and incubated for 16 h at 56 °C with [α-^{33}P]UTP (NEN, Mandel) labelled antisense probe so hybridization could occur. Following hybridization, unprotected RNA fragments were subjected to digestion (1.5 h at 30 °C) using RNase A (80 μg/mL) and RNase T1 mix (250 U/mL, Pharminigen) and protected fragments were subsequently treated with proteinase K (10 mg/mL), SDS (4%) and yeast tRNA (2 mg/mL) for 30 min at 37 °C. Isolation of protected RNA fragments was performed with phenol and chloroform extraction followed by precipitation with ammonium acetate and ethanol. Air dried samples were then reconstituted in loading buffer containing 80% formamide, heated for 3 min at 95 °C and separated on a 6% polyacrylamide sequencing gel. Phosphorimaging (Molecular Dynamics) of dried gels was used for visualization of bands and analysis.

An additional 24 male mice of the 129/SvEv strain (12 wild-type and 12 MMP-12 null) were also used for RNA analysis. MMP-12 null mice were obtained from the laboratory of Steve Shapiro (Shipley *et al.*, 1996) and a breeding colony established in the animal care facility at the University of Calgary. Briefly, eight mice from each genotype were subjected to ICH followed by either 2 or 5 days survival after which the ipsilateral striata were isolated, and the RNA extracted and used for RPA assessment of MMPs as described above. Eight mice ($n = 4$ per genotype) served as normal controls. Densitometric analysis was performed on the images obtained and expression levels determined by comparing individual MMP band intensities to L32 loading control.

Behavioural procedures

In order to assess the potential role of MMP-12 in neuronal repair and functional recovery, mice lacking MMP-12 were used. Wild-type (WT) mice ($n = 6$) and MMP-12 null ($n = 9$) mice received either ICH (collagenase) or sham (S, saline $n = 13$) surgery and were then assessed for sensorimotor function using several behavioural tests. Mice were socially housed (five per cage) and maintained on a 12-h light : 12-h dark cycle. Animals had free access to food and water, with the exception of behavioural training and test periods, during which food was restricted (4 g per day per animal) and animals were maintained at approximately 80% of original body weight. All experimental procedures were in accordance with Canadian Council for Animal Care guidelines and received prior approval by Memorial University and the University of Calgary Animal Care Committees. All behavioural assessment and histological estimates were performed by experimenters blind to group condition.

Single pellet skilled reaching

Prior to ICH, animals were trained for three weeks to reach for 20 mg food pellets (Research Diets, New Brunswick, NJ). The reaching apparatus consisted of a plexiglass box with a 5.0 mm slot situated against the front-left wall, thus forcing the animals to reach through the slot with their left (impaired) forepaw. Mice were assessed on their ability to retrieve a single food pellet situated on an elevated shelf approximately 1.0 cm outside the box. Pellets were presented one at a time and reaches were recorded with a Sony TRV-17 videocamera, and the number of successful (retrieved and eaten) and unsuccessful (any forelimb advancing through the plane of the plexiglass wall, resulting in a missed or dropped pellet) reaches were determined. Reaching performance was assessed prior to surgery and repeated at seven, 14 and 28 days following ICH. Five mice did not learn to reach and thus were excluded from statistical analyses.

Tape removal test

Under brief halothane anaesthesia, strips of tape (1/8 inch × 1 inch; Fisherbrand) were lightly placed lengthwise over the dorsal and

ventral surfaces of the forepaws. The first paw to be contacted and the latencies to remove the tape from each paw were recorded. Training consisted of five trials (one per day) and testing subsequent to ICH was carried out once weekly at six, 12, 18, 24 and 28 days after ICH.

Cylinder test of forelimb asymmetry

Animals were examined for preferential use of the ipsilateral forelimb during upright postural support. Mice were placed in a plexiglass cylinder (10 cm in diameter) on a glass table-top and video-recorded from below (via an angled mirror) for 5 min. The number of ipsilateral, contralateral and bilateral wall contacts was recorded. The percentage of bilateral contacts was assessed on each test day using the formula (100 × bilateral contacts/total forelimb wall contacts), whereas the percentage of ipsilateral contacts was assessed using the formula [100 × ipsilateral contacts/(ipsilateral contacts + contralateral contacts)].

Histological procedures

Upon completion of behavioural assessment at day 30, animals were given an overdose of sodium pentobarbitol and transcardially perfused with 0.9% saline followed by 5.0% phosphate buffered formalin. Brains were removed and placed in perfusate for 2 h, before being put into 20% sucrose solution. Brains were then frozen on dry ice, sectioned at 30 μm (every eighth section was collected) and stained with cresyl violet. The volume of haemorrhagic tissue injury was estimated using unbiased stereological procedures (Howard & Reed, 1998) and the volume of histologically intact tissue in the affected striatum was compared to the striatum in the opposite undamaged hemisphere.

Similarly, 16 mice (eight WT and eight MMP-12 null) were subjected to ICH followed by 5 days survival. Subsequently, the animals were given an overdose of sodium pentobarbitol and transcardially perfused as described above. Brains remained *in situ* for 24 h and were then embedded in paraffin prior to sectioning. Six micron sections were cut and processed for Iba1 immunohistochemistry to label microglia/macrophages. Briefly, sections were rehydrated and rinsed with PBS followed by antigen retrieval using 10 mM sodium citrate buffer (pH 6.5). Primary antibody (Chemicon) was diluted 1 : 500 and sections incubated overnight at 4 °C. Biotinylated anti-rabbit IgG was used for the secondary antibody and staining was visualized with ABC using DAB as the substrate. Sections were analysed blind for the degree of microglial/macrophage activation determined by the morphology and density of the Iba1 labelled cells as previously described (Wells *et al*., 2003). Both ispilateral and contralateral hemispheres were evaluated.

Statistical analysis

Behavioural data were analysed using repeated measures ANOVA and *posthoc* group comparisons were made using Fisher's PLSD. Control animals (WT and MMP-12 null mice) did not differ in behavioural performance so were pooled into a single group (S) for statistical analyses. The volume of remaining histologically intact tissue was analysed using unpaired Student *t*-tests. MMP expression levels were analysed using ANOVA followed by *posthoc* comparison's using the Tukey–Kramer multiple comparison test. Iba1 immunoreactivity was analysed using Mann–Whitney test for nonparametric data. For all statistical analyses $P < 0.05$ was considered significant.

Results

RNase protection assay

The MMP multiprobe set identifies nine members of the MMP family (MMP-2, -3, -7, -9, -10, -11, -12, -13 and -14). In addition, the ribosomal protein L32 serves as an internal loading control. The data are presented in Fig. 1. The expression of several MMPs transcripts (MMP-3, -12 and -14) is up-regulated following ICH. Most notable, however, is the substantial increase in transcripts encoding MMP-12. While below the level of detection in normal brain, MMP-12 levels increase to detectable levels around 24 h after ICH with peak expression occurring at 5 days.

Single pellet skilled reaching

Figure 2 depicts performances in the reaching task throughout the duration of the experiment. All mice were trained in this task prior to induction of ICH and data are presented as percentages, where an animal's scores in the post tests are normalized to its pre-training score. Seven days following ICH, the performance of all groups dropped below 100% including the S operated group. Repeated measures ANOVA confirmed a significant effect of group ($F_{2,20} = 4.20$; $P = 0.034$), test day ($F_{3,60} = 15.2$; $P = 0.002$) and a group by test day interaction ($F_{6,60} = 2.88$; $P = 0.02$). Reaching performance was impaired in both ICH groups compared to S operated controls ($P < 0.05$) with a trend for further decreased performance in the WT compared to MMP-12 null mice. Fourteen days after ICH the performance of MMP-12 null mice, while lower than S, was not significantly different. In contrast, WT mice remained significantly impaired compared to S ($P < 0.05$). Furthermore, there was a significant difference between WT and MMP-12 null mice ($P < 0.05$). Twenty-eight days following ICH, none of the animals had recovered to pre-training levels, although they did exhibit gradual improvements throughout the testing period. While MMP-12 null mice achieved performance levels similar to S operated mice, WT mice remained significantly impaired in performing this task ($P < 0.05$).

Tape removal test

The data generated from the tape removal task is presented in Fig. 3. Baseline scores were obtained during the training sessions. Data are expressed as latencies with impairments determined by subtracting the ipsilateral (intact) paw performance from the contralateral (impaired) paw performance. Repeated measures ANOVA showed a significant effect of group ($F_{2,26} = 9.7$; $P = 0.0008$), test day ($F_{9,234} = 3.77$; $P = 0.0002$) and a group by test day interaction ($F_{18,234} = 1.94$; $P = 0.02$). *Posthoc* tests revealed no significant differences between any of the groups during the training period. However, 7 days after ICH, both WT and MMP-12 null groups demonstrated significant impairment in this task such that latencies were significantly elevated relative to the S group (401 ± 104 and 368 ± 96 s for WT and MMP-12 null, respectively, vs. 2.7 ± 14 s for S). Recovery of performance on this task was modest with both groups remaining significantly different from sham at all time points assessed. However, after 28 days, there was a strong trend for improved performance in the MMP-12 null mice relative to WT ($P = 0.08$).

Cylinder test of forelimb asymmetry

Figure 4 depicts the data accumulated from the cylinder test of forelimb asymmetry. Performance in this task was assessed by

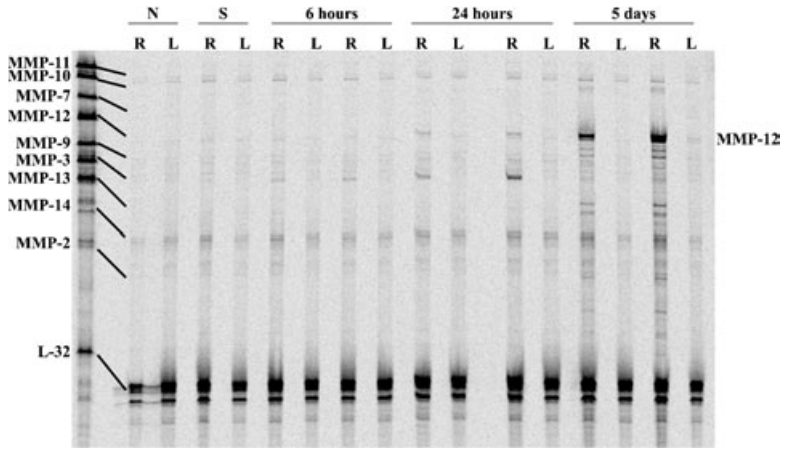


FIG. 1. RNA transcript levels of several MMPs are up-regulated after collagenase induced intracerebral haemorrhage. RNA levels of nine MMP family members were assessed 6 h, 24 h and 5 days after injury in both ipsilateral (affected) and contralateral (unaffected) striatum. Six hours after injury faint bands corresponding to MMP-3 are visible in the ipsilateral striata. Peak expression of MMP-3 occurs at 24 h. MMP-14 is constitutively expressed in the brain as bands are evident in normal mice as well as saline injected controls in both ipsilateral and contralateral striata. There appears to be increased expression of this MMP as bands are more intense in ipsilateral striata at 5 days after injury. Finally, RNA transcript encoding for MMP-12 is increased 24 h after injury with peak expression at 5 days. Of all the MMPs examined in this assay, MMP-12 levels were most highly up-regulated leading to a more focused assessment of the role of MMP-12 in ICH pathology. This data are representative of two separate experiments where a total of four animals per group were assessed.

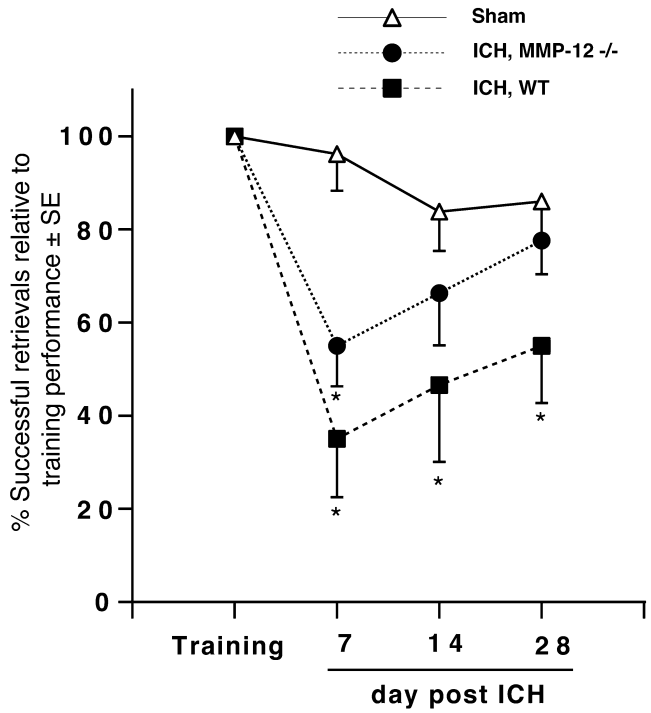


FIG. 2. MMP-12 protease contributes to continued functional deficits in single pellet skilled reaching. Prior to injury, all animals were trained in the reaching task and post-injury scores have been normalized to pre-injury scores. Seven days after injury all groups including sham operated controls exhibited decreases in performance. Repeated measures ANOVA with *posthoc* analysis indicate that both MMP-12 null and WT mice performed significantly worse than sham controls ($P < 0.05$) with WT mice exhibiting the lowest scores. By 14 days post-injury the MMP-12 null mice improved to a level that was not significantly different than sham while WT mice, although improved from day 7 remained significantly impaired compared to sham ($P < 0.05$) and performed significantly worse than MMP-12 null mice ($P < 0.05$). At the end of the experiment, 28 days after ICH, MMP-12 null mice achieved performance levels that were similar to the sham operated controls. In contrast, functional deficits persisted in the WT mice ($P < 0.05$). *Significantly different from S; †significantly different from MMP-12–/–.

examining both the number of bilateral wall contacts (as a percentage of total forelimb contacts), as well as the number of ipsilateral wall contacts (as a percentage of total single forelimb contacts). Prior to ICH, all mice tended to preferentially make bilateral wall contacts when rearing in the cylinder, with relatively equal use of either forepaw for single limb contacts. Statistical examination of bilateral limb use revealed significant effects of group ($F_{2,22} = 6.4$; $P < 0.010$ and test day ($F_{3,66} = 5.87$; $P < 0.002$, but no interaction ($F_{6,66} = 0.802$; $P = 0.57$). ICH resulted in an early and marked decrease in bilateral contacts made by WT mice (significantly different from S at seven and 14 days post-ICH) which recovered by 28 days. Interestingly, MMP-12 null mice did not exhibit any decline in the number of bilateral contacts made and were not significantly different from sham animals at any time.

With respect to ipsilateral wall contacts, repeated measures ANOVA revealed an effect of group ($F_{2,22} = 3.7$; $P < 0.05$) and test day ($F_{3,66} = 5.89$; $P < 0.002$) but no interaction ($P = 0.22$). Reliance on the intact forelimb was increased in all ICH animals compared to training. Performance in the MMP-12 null mice was significantly different from the S group at day 7 but not at day 14. In contrast, WT mice showed a significant preference for the ipsilateral paw at both seven and 14 days post-ICH compared to S and MMP-12 null ($P < 0.05$) indicative of delayed recovery in WT mice. This difference was not maintained and none of the groups were significantly different at the end of the study 28 days post-ICH.

Histology

Histological analysis of striatal tissue indicates that there are no differences between the WT mice and mice lacking MMP-12 as determined by the percentage of remaining intact striatal tissue (Fig. 5).

No evidence of compensation by other MMPs

In order to rule out the possibility that other MMPs may be up-regulated in an attempt to compensate for the lack of MMP-12,

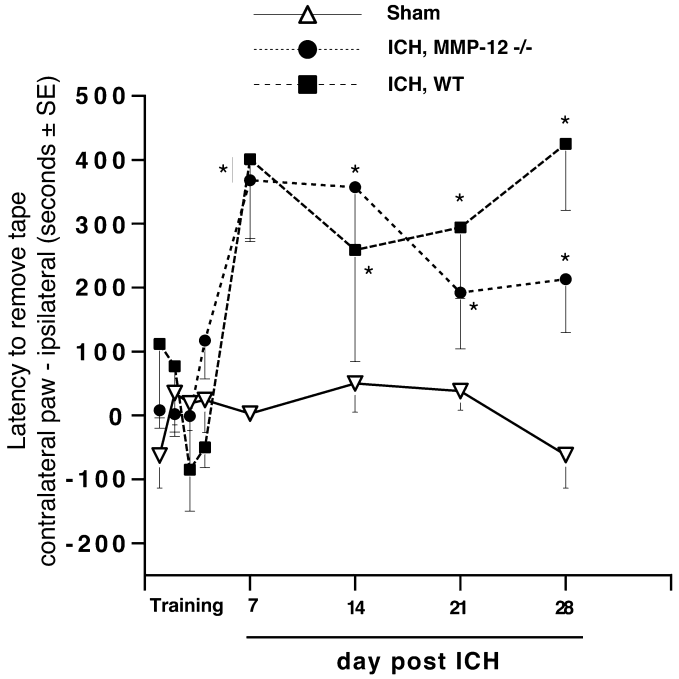


FIG. 3. MMP-12 protease increases latencies in the tape removal test. All animals were pre-trained prior to injury and baseline scores obtained. Impairments were determined by subtracting the ipsilateral (intact) paw performance from the contralateral (impaired) paw performance. Statistical analyses indicated that there were no differences in removal latencies between any of the groups throughout the training period. Seven days after ICH, both WT and MMP-12 null mice exhibited significant impairments in this task with elevated latencies of 401 ± 104 and 368 ± 96 s, respectively, compared to 2.7 ± 14 s for sham operated controls ($P < 0.05$). Performance by both WT and MMP-12 null groups continued to be poor throughout the testing period and both groups remained significantly different from sham at all time points. However, there was a strong trend towards improved performance in the MMP-12 null mice relative to WT ($P = 0.08$). *Significantly different from S.

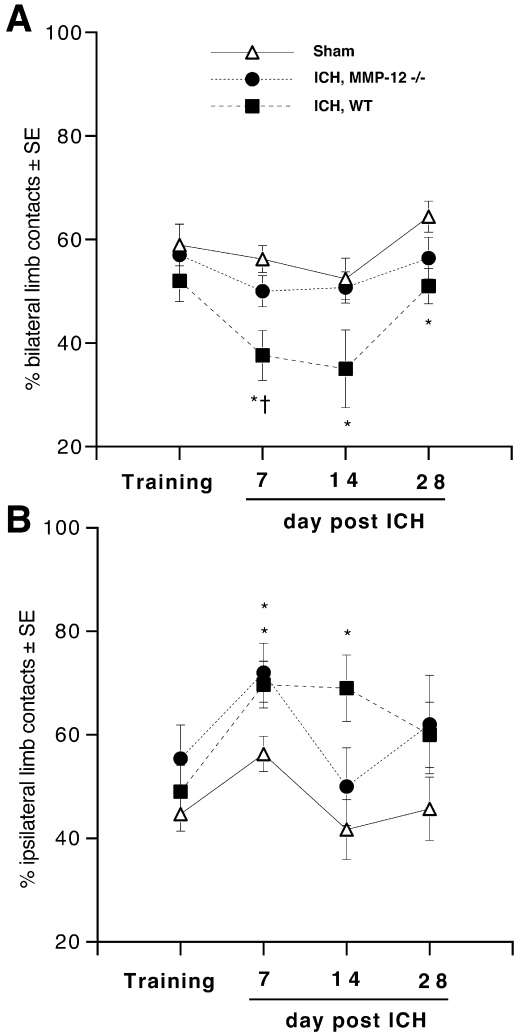


FIG. 4. MMP-12 protease alters forelimb preference in the cylinder test of forelimb asymmetry early after ICH. In A the number of bilateral wall contacts were examined and data are expressed as a percentage of total forelimb contacts. Baseline testing prior to ICH indicates that all mice, while displaying equal use of both forepaws when making single contacts, preferentially make bilateral wall contacts when rearing in the cylinder. Seven days after ICH, WT mice show a marked decrease in the percentage of bilateral wall contacts. The data collected from WT animals at seven and 14 days after ICH are significantly different from S ($P < 0.05$) but there is recovery by 28 days. ICH did not alter the behaviour of MMP-12 null mice as they were not significantly different from S animals at any time. In B the number of ipsilateral wall contacts was examined and data are expressed as a percentage of total single forelimb contacts. Both groups exhibited a reliance on the intact forelimb following ICH. MMP-12 null mice were significantly different from S at day 7, whereas WT mice demonstrated a significant preference for the ipsilateral paw at both seven and 14 days post-ICH ($P < 0.05$ vs. S and MMP-12 null) which subsequently resolved by 28 days. *Significantly different from S; †significantly different from MMP 12–/–.

RPA was performed on RNA isolated from WT and MMP-12 null mice. The results are presented in Fig. 6. Statistical analysis revealed that there were no significant differences between WT and MMP-12 null mice under either normal conditions, or 2 and 5 days after ICH for all the MMPs examined. Furthermore, the up-regulation of MMP-12 that occurs 5 days after injury was confirmed in this strain of mice ($P < 0.01$).

Histological assessment of microglia/macrophages

Iba1 immunostaining was used to evaluate the pattern of microglia/macrophage recruitment and activation following ICH. Inspection of the ipsilateral hemisphere showed that 5 days after injury there was an increase in recruitment of Iba1-positive cells having an amoeboid appearance consistent with macrophages bordering the lesion (Fig. 7A and B). The lesion itself was largely devoid of any labelling. Adjacent to this rim of rounded cells are Iba1-positive cells exhibiting the morphology of activated microglia with intensely stained shortened and thickened processes (Fig. 7C and D). Finally as one moves away from the lesion the labelled cells appear to be less activated displaying the morphology of resting or quiescent microglia with thin, highly ramified processes (Fig. 7E and F). Blinded analysis of the tissue sections revealed a significant difference in the density of Iba1 immunostaining, particularly the rounded cells, between WT and MMP-12 null mice as indicated in Fig. 7G ($P < 0.05$).

There was no evidence of macrophage recruitment or microglial activation in the contralateral hemisphere in any of the mice (data not shown).

Discussion

MMPs have been implicated in playing a pathophysiologic role in both animal and human studies of ischemic (Anthony *et al.*, 1997; Romanic *et al.*, 1998; Gasche *et al.*, 1999; Heo *et al.*, 1999) and

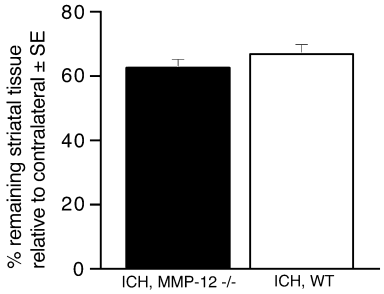


FIG. 5. The lack of MMP-12 does not alter the amount of tissue remaining after ICH. The percentage of remaining intact striatal tissue (expressed as a percentage of contralateral striatum) indicates that there were no significant differences between the WT and MMP-12 groups.

haemorrhagic stroke (Rosenberg & Navratil, 1997; Abilleira *et al*., 2003) as well as haemorrhagic transformation (Heo *et al*., 1999; Lapchak *et al*., 2000; Montaner *et al*., 2001b; Sumii & Lo, 2002; Castellanos *et al*., 2003). Currently, there are 23 known MMPs subdivided into different classes dependent on their substrate specificity. The expression of MMPs in CNS pathology appears to be quite variable as to which MMP member is expressed and also when it is expressed. Furthermore, the functional consequence of such MMP expression in CNS pathology remains to be elucidated.

The most studied MMPs have been the gelatinases, MMP-2 and MMP-9, in large part because of the relative ease of determining the presence of these MMPs using the method of gelatin zymography. Using RNase protection assay, allowing for the simultaneous assessment of nine MMP family members, we found alterations in the RNA levels of several MMPs. Most notable was the increased expression of MMP-12 mRNA. The up-regulation of MMP-12 expression was detectable at 24 h post-ICH in the affected hemisphere but not in the intact hemisphere. Peak expression occurred at 5 days post-ICH. The lack of increased MMP levels in the contralateral uninjured hemisphere is consistent with previous reports (Rosenberg & Navratil, 1997). It is unknown whether the expression of MMP-12 remains high after the times assessed in this study.

The specific role of MMP-12 in ICH has not been previously tested. MMP-12 is a metalloelastase that can act on many substrates including collagen IV, casein, elastin, fibronectin, gelatin, laminin, proteoglycan link protein and vitronectin (Yong *et al*., 2001). It has been implicated in other disease processes including emphysema (Hautamaki *et al*., 1997; Horton *et al*., 1999) and abdominal aortic aneurysms (Curci *et al*., 1998) where it has been shown to play a prominent role in the pathology of these diseases. Recently MMP-12 has been implicated in CNS pathology including multiple sclerosis (MS) (Vos *et al*., 2003), spinal cord injury (Wells *et al*., 2003) and rat ICH (Power *et al*., 2003). With respect to MS, Vos and colleagues showed that a high number of MMP-12 positive phagocytic macrophages were present in active demyelinating lesions with lower numbers present in chronic active demyelinating lesions and inactive lesions but were not present in control tissue (Vos *et al*., 2003). Furthermore, in a mouse model of spinal cord injury, MMP-12 mRNA levels were found to be up-regulated and increased expression of MMP-12 was associated with increased microglial activation, macrophage infiltration and blood–spinal barrier permeability in WT. In contrast, these were reduced in mice deficient for MMP-12, which subsequently demonstrated improved functional recovery (Wells *et al*., 2003). Finally, in a rat model of ICH, Power *et al*., (2003) demonstrated that minocycline, an agent with anti-inflammmatory properties resulted in improved neurobehavioural outcome compared to untreated controls and that this was correlated to reduced MMP-12 levels in the brain; the possible detriment of MMP-12 was not directly tested in this rat study.

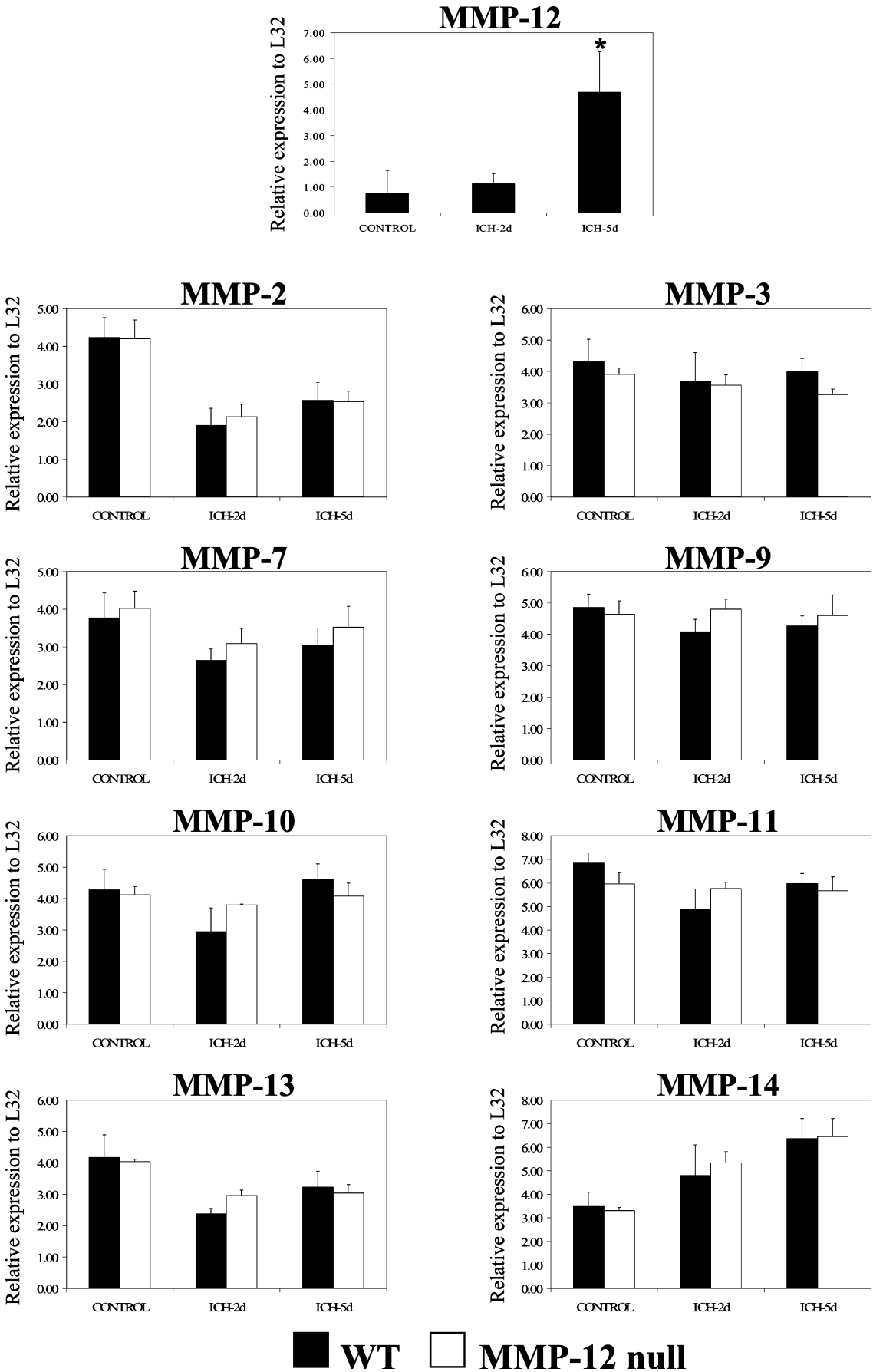
In the current report, and by using mice lacking MMP-12, we have provided further evidence that MMP-12 expression is detrimental following ICH. While the histological analysis did not reveal any significant differences between the MMP-12 null and WT mice, there were significant differences in functional outcome. Our assessments of lesion volume measured only the remaining intact striatal tissue. Autoradiography and magnetic resonance imaging (MRI) studies have previously been performed in rats and document the oedema formation produced in this model of ICH (Rosenberg *et al*., 1990a; Del Bigio *et al*., 1996). They have shown that oedema formation in this model is extensive and can spread along the white matter to affect other brain regions. For example, Rosenberg *et al*. (1990a) demonstrated that fluid contributing to oedema was able to flow along white matter fibre tracts into the posterior regions overlying the hippocampus and then via the hippocampal commissure enter the nonhemorrhagic or un-injected hemisphere. It is possible that axonal damage, undetectable when assessing striatal regions alone, occurs in this model and may explain differences in functional outcome between MMP-12 null and WT mice. Future work, using more sophisticated analysis (e.g. myelin stains, electrophysiological or MRI) would be required to test this idea.

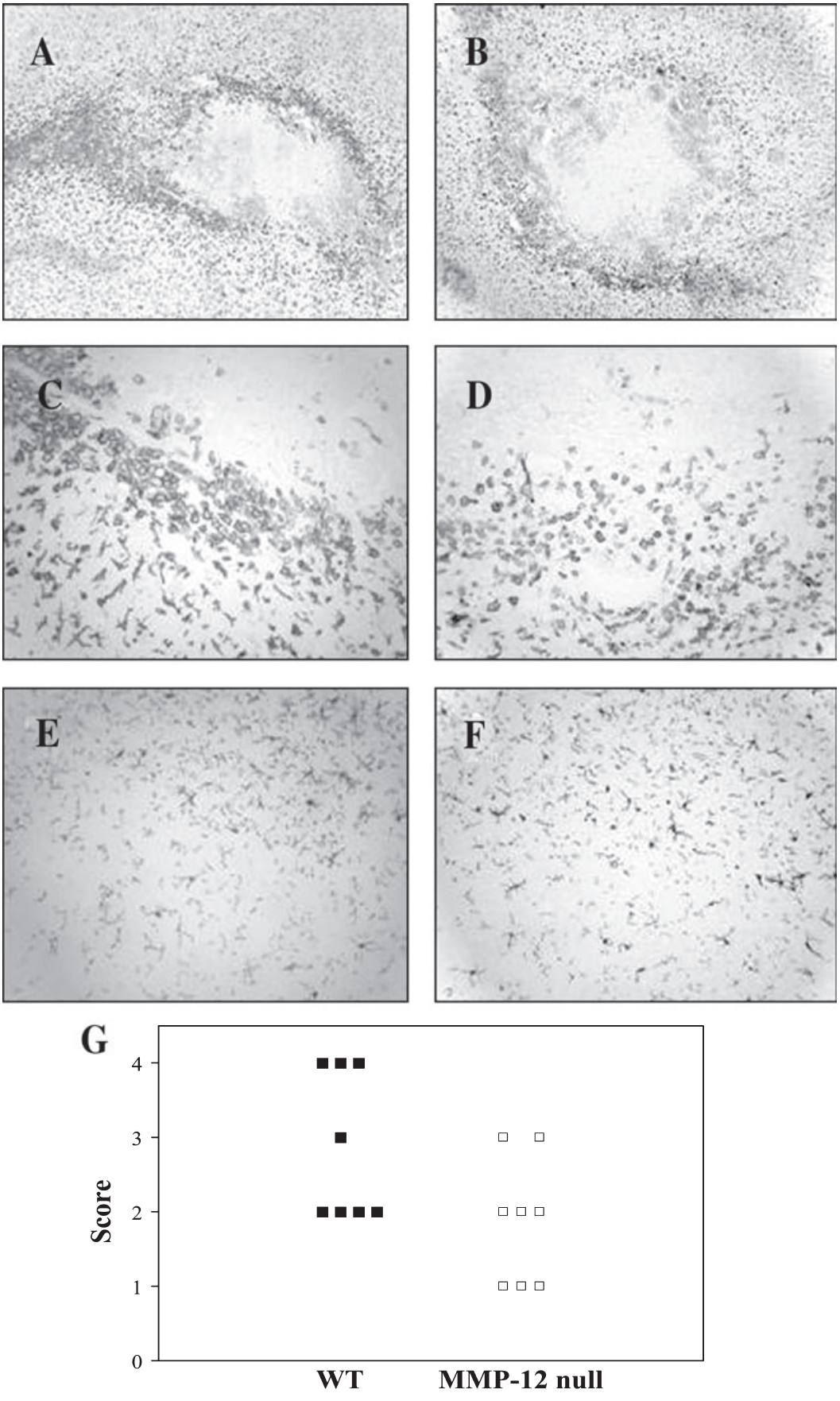
In contrast and perhaps more relevant to the clinical situation, functional outcome, as assessed using several behavioural tasks, was significantly different between the two groups. The data indicate that MMP-12 deficient mice have significantly improved performance in single pellet skilled reaching and forelimb asymmetry with a trend towards improved recovery in tape test removal. Single pellet skilled reaching and the forelimb asymmetry tasks are both indices of sensorimotor function while deficits in the tape removal test are mainly sensory (Schallert & Whishaw, 1984; Schallert *et al*., 2000). Improvement in all tasks indicates that MMP-12 deficiency is having a global effect on CNS function of the infarcted hemisphere and that MMP-12 expression after trauma is detrimental. Furthermore, the fact that MMP-12 expression occurs early after injury indicates that MMP-12 is most likely contributing to the development of secondary injury rather than impeding recovery.

The expression profiles of the MMPs were also assessed in WT and MMP-12 null mice under normal conditions and following ICH. In this strain of mice, MMP-12 levels were significantly elevated 5 days post-injury confirming our original observations. Furthermore, and more importantly, we have demonstrated that in mice lacking MMP-12 there are no compensatory changes in the expression of the other MMPs examined (Fig. 6) suggesting that the differences in outcome between the two genotypes reflects the status of MMP-12.

MMP-12 is highly expressed in cells of the macrophage lineage (Banda & Werb, 1981; Shapiro *et al*., 1993). One of the features of

FIG. 6. Other MMPs do not compensate for the loss of MMP-12. Comparisons of MMP expression levels between WT and MMP-12 null mice under normal conditions as well as following injury indicate that other MMPs are not compensating for the lack of MMP-12. While we did see a significant increase in MMP-12 levels in WT mice at 5 days injury ($P < 0.01$), consistent with results in Fig. 1, there were no differences between WT and MMP-12 null mice in the other eight MMPs examined.


MMP-12
Relative expression to L32
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
CONTROL
ICH-2d
ICH-5d
MMP-2
MMP-3
MMP-7
MMP-9
MMP-10
MMP-11
MMP-13
MMP-14
WT
MMP-12 null


A
B
C
D
E
F
G
Score
4
3
2
1
0
WT
MMP-12 null

ICH is the disruption of the blood brain barrier (BBB). As a result, cells from the periphery including macrophages are able to enter the CNS. Once inside, the expression of MMP-12 by macrophages could be deleterious by contributing further to BBB permeability. Evidence to support this type of role comes from a recent paper looking at the role of MMP-12 in spinal cord injury (Wells *et al*., 2003). In this study, MMP-12 produced by macrophages was found to be highly up-regulated following compression injury and followed the same temporal pattern of expression as in ICH. Furthermore, in mice lacking MMP-12 protease, improved functional outcome was associated with decreased blood-spinal permeability post-SCI. Alternatively or in combination with potential effects on BBB, MMP-12 within the CNS may be directly damaging by degrading extracellular matrix (ECM) components, by facilitating the entry of peripheral cells into the parenchyma or by the processing of pro-imflammatory molecules into inflammatory molecules and thereby promote CNS inflammation, activities which have been described for other MMPs (Yong *et al*., 2001).

In the present study, mononuclear phagocytes were also examined by Iba1 immunoreactivity 5 days after ICH at a time when MMP-12 transcript levels are elevated. The data indicate that while the patterns of macrophage infiltration and microglial activation are similar in WT and MMP-12 null mice, there is a significant difference in the degree of this response. The density of rounded cells bordering the lesions of WT mice was higher than that seen in MMP-12 null mice (Fig. 7). The presence of these cells in higher numbers may be contributing to the behavioural differences observed between the two genotypes and further implicate MMP-12 in the pathophysiology of ICH. Future work regarding the specific mechanisms of MMP-12 in secondary injury after ICH is required.

Other MMPs were also up-regulated after ICH including MMP-3 and MMP-14 (Figs 1 and 6). The increase in MMP-14 mRNA has, to our knowledge, not been reported previously and its precise role in ICH remains to be elucidated. In the present study MMP-3 RNA was increased 24 h after ICH. A previous study in rats also reported increased MMP-3 levels at 24 h (Power *et al*., 2003). Furthermore, in a recent paper by Alvarez-Sabin and colleagues, MMP-3 levels, as measured by ELISA, were increased at 24 and 48 h after ICH in humans. Moreover, MMP-3 levels at 24 h were correlated to residual scar volume measured at 3 months as well as mortality (Alvarez-Sabin *et al*., 2004). While this study also implicates MMP-3 in the pathophysiology of ICH we did not find any differences in the expression of this MMP between WT and MMP-12 null mice indicating that it is not contributing to the behavioural differences observed in these mice.

Interestingly, we did not find markedly increased expression in either MMP-2 or MMP-9. The up-regulation of MMP-2 expression in stroke has been noted previously (Rosenberg *et al*., 1996; Clark *et al*., 1997; Romanic *et al*., 1998; Heo *et al*., 1999; Abilleira *et al*., 2003). However, there is some controversy as to whether or not this MMP is relevant to stroke pathology. In one study, Asahi and colleagues (Asahi *et al*., 2001) induced focal ischemia in MMP-2 null mice and WT controls. Measuring several parameters, they showed no difference in blood flow reduction, neurological outcome and lesion volumes and concluded that MMP-2 does not play a significant role in stroke pathology; this is in contrast to MMP-9. While we did not see a pronounced increase in MMP-9 mRNA levels, this MMP has been strongly implicated in stroke pathology in both animal models (Del Bigio *et al*., 1996; Rosenberg & Navratil, 1997; Romanic *et al*., 1998) as well as human cases (Montaner *et al*., 2001a; Abilleira *et al*., 2003; Castellanos *et al*., 2003). For example, in human ICH the increase in MMP-9 levels as measured by ELISA was associated with peri-haematomal oedema and neurological worsening (Abilleira *et al*., 2003). Furthermore, treatments with MMP inhibitors (Rosenberg & Navratil, 1997) and more compellingly MMP-9 neutralizing antibodies (Romanic *et al*., 1998) have demonstrated significant reductions in brain oedema and brain injury, respectively. The most studied mechanism of MMP-9 action has been with respect to the BBB where it has been shown to contribute to increased permeability and subsequent oedema formation (Rosenberg *et al*., 1998; Fujimura *et al*., 1999; Gasche *et al*., 1999; Pfefferkorn & Rosenberg, 2003). The reason for the discrepancy between our study and previous reports in terms of MMP-2 and MMP-9 is not known but may be due to differences in species studied or how the MMPs were studied (RNA vs. protein). However, it is important to note that the other studies did not assess MMP-12, and because the current data demonstrate higher expression of MMP-12 compared to MMP-9, we propose that MMP-12 may be key to outcome from ICH injury in humans.

Finally, we did not observe elevations of MMP-7 in contrast to its increase noted in rat ICH injury (Power *et al*., 2003). MMP-7 is an MMP that is thought to be species-specific, in that its elevation is noted in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis, but only in rats (Clements *et al*., 1997) and not in mice (Pagenstecher *et al*., 1998; Brundula *et al*., 2002).

In summary, this work demonstrates an adverse role for MMP-12 in ICH pathology. MMP-12 expression is up-regulated after ICH and in its absence mice exhibit improved sensorimotor function. MMP-12 can now be added to the list of MMPs demonstrating pathological roles in secondary injury development after ICH and this finding further supports the contention that MMP inhibitors may be a useful therapeutic strategy in stroke.

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Abbreviations

BBB, blood brain barrier; CNS, central nervous system; ICH, intracerebral haemorrhage; MMP, matrix metalloproteinase; S, sham; WT, wild-type.

FIG. 7. Microglia/macrophage infiltration is reduced in MMP-12 null mice. In panels A and B, which are Iba1 stained rounded cells bordering the lesion (likely macrophages), there was decreased density in MMP-12 null mice compared to the WT animals. Panels C, D, E and F demonstrate the changes in morphologies observed as distance from lesion border increases. Panel C and D are taken from the lesion border and show a rim of darkly stained rounded cells characteristic of macrophages (arrowheads) adjacent to the lesion itself, which is largely devoid of Iba1 immunoreactivity. The density of these cells increased in WT (panel C) compared to MMP-12 null (panel D). Directly adjacent to this rim is a second rim of labelled cells exhibiting morphology characteristic of activated microglia with darkly stained processes that are short and thick (arrows). Still further from the lesion site (panels E and F) the morphology of the labelled cells resembles that of resting or quiescent microglia. Data comparing the relative densities of Iba1 labelled cells in WT and MMP-12 null mice are presented in panel G. Statistical analysis revealed a significant difference in the infiltration of mononuclear phagocytes between WT and MMP-12 null mice ($P < 0.05$).

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