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Bone marrow-derived cells are the major source of MMP-9 contributing to blood-brain barrier dysfunction and infarct formation after ischemic stroke in mice

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Abstract

Matrix metalloproteinase (MMP)-9 has been shown to contribute to blood-brain barrier (BBB) disruption, infarct formation, and hemorrhagic transformation after ischemic stroke. The cellular source of MMP-9 detectable in the ischemic brain remains controversial since extracellular molecules in the brain may be derived from blood. We here demonstrate that bone marrow-derived cells are the major source of MMP-9 in the ischemic brain. We made bone marrow chimeric mice with MMP-9 null and wild-type as donor and recipient. After 90 min of transient focal cerebral ischemia, MMP-9 null mice receiving wild-type bone marrow showed comparable outcomes to wild-type in brain MMP-9 levels and BBB disruption (endogenous albumin extravasation) at 1 h post-reperfusion and infarct size at 24 h post-reperfusion. In contrast, wild-type animals replaced with MMP-9 null bone marrow showed barely detectable levels of MMP-9 in the ischemic brain, with attenuations in BBB disruption and infarct size. MMP-9 null mice receiving wild-type bone marrow showed enhanced Evans blue extravasation as early as 1 h post-reperfusion compared to wild-type mice replaced with MMP-9 null bone marrow. These findings suggest that MMP-9 released from bone marrow-derived cells influences the progression of BBB disruption in the ischemic brain.

Keywords

Injury; Ischemia; Neuroinflammation; Gelatinase; Blood-brain barrier

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1. Introduction

Blood-brain barrier (BBB) disruption after cerebral ischemia potentiates brain injury by several mechanisms including vasogenic edema, exposure of parenchyma to intravascular constituents, diminished cerebral blood flow, and hemorrhagic conversion (del Zoppo and Hallenbeck, 2000). Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of post-ischemic BBB disruption (Mun-Bryce and Rosenberg, 1998). Among MMPs, MMP-9 is a major component in post-ischemic BBB disruption. Enhanced MMP-9 expression and activity were demonstrated in ischemic brain (Fujimura et al., 1999; Gasche et al., 1999). MMP inhibitors and MMP-9 gene deletion attenuated BBB disruption and brain tissue infarction after ischemia (Asahi et al., 2000; Asahi et al., 2001; Jiang et al., 2001). Clinical studies have shown a correlation between plasma MMP-9 levels and the rate of hemorrhagic transformation in human stroke (Montaner et al., 2001; Castellanos et al., 2007).

Although MMP-9 is detectable in the brain after stroke, its cellular source remains under debate. Parenchymal elements (endothelium, glia, or neurons) have been shown positive for MMP-9 immunostaining after ischemia (Fujimura et al., 1999; Asahi et al., 2001; Maier et al., 2004). Since MMP-9 functions as a protease after being secreted from cells, the location of MMP-9 immunoreactivity does not necessarily reflect the cells releasing MMP-9. In several peripheral organs, MMP-9 derived from bone marrow-derived cells (BMDC) is functionally significant (Vu et al., 1998; Coussens et al., 2000; Pyo et al. 2000). BMDC have also been suggested to be an important source of MMP-9 in the brain after ischemia (Justicia et al., 2003; Gidday et al., 2005; McColl et al., 2008). Neutropenia or intercellular adhesion molecule (ICAM)-1 blocking antibody attenuated the level of MMP-9 in the ischemic brains of rats at 24 h after 60 min of transient middle cerebral artery occlusion (MCAO) (Justicia et al., 2003). Gidday et al. (2005) demonstrated, by using chimeric mice with MMP-9 knockout and wild-type animals, that leukocytes are the major source of MMP-9 in the ischemic brain at 24 h after 2 h of transient MCAO. McColl et al. (2008) showed that neurovascular gelatinolytic activity is mediated by neutrophil-derived MMP-9 in the brains of interleukin-1 β -challenged mice after MCAO. Conversely, Maier et al. (2004) showed dissociation in the spatial and temporal relationship between MMP-9 immunostaining and neutrophil infiltration. Harris et al. (2005) failed to detect an influence from neutrophil depletion on MMP-9 levels in ischemic rat brain at 24 h after 3 h of MCAO. Thus, infiltrating leukocytes may not be the sole source of MMP-9 in the ischemic brain at later reperfusion times such as 24 h or longer.

Changes in BBB dysfunction after ischemia/reperfusion have multiple phases (Yang and Betz, 1994; Belayev et al., 1996). Little is known about the functional significance of MMP-9 in the initiation phase of BBB dysfunction caused by ischemia/reperfusion. Here, we show that BMDC are the major cellular source of MMP-9 detectable in the ischemic brain tissue after 90 min of MCAO and 1 h of reperfusion. In addition, MMP-9 released from BMDC contributes to BBB dysfunction at 1 h after reperfusion and subsequent infarct formation at 24 h.

2. Results

2.1. BMDC contribute to MMP-9 activity in ischemic brain

To determine the source and nature of the MMP-9 like gelatinase activity (100 kDa) in the brain after ischemia/reperfusion, bone marrow chimeric mice were developed from combinations of MMP-9 KO and WT mice as donors and recipients (Fig. 1). In this manner, WT/KO mice contained the MMP-9 gene only in BMDC while KO/WT mice contained the MMP-9 gene in brain parenchymal cells (including neurons, astroglia, and vessels) with a minimal number of BMDC expressing the MMP-9 gene.

Complete blood count showed that 6 weeks of recovery after irradiation and bone marrow transplantation were sufficient to repopulate blood cells (Table 1). We also confirmed that the current conditions were efficient to reconstitute bone marrow since immunocytochemical analysis showed that 86.9% of nucleated blood cells were MMP-9 positive in WT/KO mice, whereas 10.5% of nucleated cells were MMP-9 positive in KO/WT (Fig. 3 and Table 2). In addition, 88.3 ± 2.9 % of nucleated cells were mRFP1 positive in RFP/KO mice ($n = 3$). Moreover, spleens from the WT/KO mice showed comparable MMP-9 like gelatinase levels to those from WT and spleens from the KO/WT mice showed minimal MMP-9 like gelatinase levels as seen in the KO animals (Fig. 4A and C). These findings also confirmed that the gelatinase activity (100 kDa) in the spleen was MMP-9.

The ischemic brain hemispheres of WT/KO mice demonstrated comparable MMP-9 like activity levels to those of WT or WT/WT at both 1 h and 24 h after reperfusion following 90 min of MCAO. In contrast, KO/WT, KO, and KO/KO mice were devoid of the activity at both time points, confirming that the gelatinase (100 kDa) detectable in the ischemic brain was associated with MMP-9 (Fig. 4B and C). At 1 h, a lower band at 75 kDa was detectable in both hemispheres in WT and WT/KO but not in KO/WT and KO (Fig. 4B), suggesting that this 75 kDa activity was also associated with MMP-9. The intensities of the lowest band (65 kDa), presumably reflecting MMP-2 activity, were similar between the ischemic and contralateral hemispheres across the four groups, indicating that MMP-2 was not affected by MMP-9 gene deletion (Fig. 4B and C). Immunoblotting with anti-MMP-9 antibodies detected a single band at 100 kDa in the ischemic hemispheres and spleens from WT/WT and WT/KO but not from KO/WT and KO/KO mice, confirming that the gelatinase activity was MMP-9 (Fig. 4D).

In situ zymography using brain sections treated with DQ-gelatin showed fluorescent signals in ischemic area from WT/WT and WT/KO mice at 24 h after reperfusion (Fig. 5A and B), primarily associated with neuronal nuclei. Consistent with gel zymography and immunoblotting (Fig. 4C and D), KO/WT and KO/KO mice showed minimal levels of the fluorescent signal (Fig. 5C and D) at the same time point. These findings suggested that gelatinase activity detectable in the ischemic brain was derived from BMDC. In addition, *in situ* zymography was specific for MMP-9 activity under the current experimental conditions since the signal was barely detectable in KO mice. Confocal laser scanning microscopy using brain sections from WT/KO mice showed MMP-9 activity associated mainly with neurons and microvasculature to a lesser extent (Fig. 5E), whereas MMP-9 immunostaining was seen in leukocyte-like cells and microvasculature (Fig. 5F). KO/WT and KO/KO mice showed minimal MMP-9 immunostaining in the ischemic brain (data not shown).

2.2. BMDC-derived MMP-9 contributes to BBB dysfunction during early reperfusion period

We investigated whether MMP-9 contributes to BBB disruption as early as 1 h after post-ischemic reperfusion by measuring endogenous albumin levels in the brain parenchyma. Under non-ischemic conditions, the albumin extravasation was low and there was no difference between WT and KO animals (Fig. 6A). After ischemia/reperfusion, there was a significant increase (2.5 fold) in the brain albumin level at both 1 and 18 h after reperfusion in wild-type animals (Fig. 6B and C). The contralateral hemispheres after ischemia/reperfusion also showed considerably higher levels of albumin (2 fold) compared to non-ischemic sham, suggesting that the BBB was affected even on the contralateral side. At 1 h after reperfusion, WT/KO mice showed similar levels of albumin extravasation to WT animals. Conversely, KO/WT mice showed attenuated levels of albumin extravasation similar to those in KO mice (Fig. 6B). At 18 h after reperfusion, KO mice showed significantly attenuated levels of albumin extravasation in both ischemic and contralateral hemispheres compared with WT (Fig. 6C). Separate experiments measuring Evans blue extravasation confirmed that BBB disruption on the ischemic side was significantly enhanced in WT/KO mice compared with KO/WT at 1 h

after reperfusion (Fig. 6D). These findings suggested that MMP-9 from BMDC is a critical component of the BBB dysfunction after ischemia/reperfusion.

2.3. BMDC-derived MMP-9 contributes to brain tissue injury

Finally, we examined whether MMP-9 derived from BMDC is responsible for brain tissue injury at 24 h after reperfusion following 90 min of MCAO. As brain irradiation and bone marrow transplantation could alter BBB and immune-responses, KO/KO and WT/KO mice served as a control. WT/KO mice showed comparable infarct volume to WT/KO mice. KO/KO and KO/KO mice demonstrated significantly smaller infarcts compared with WT/KO animals (Fig. 6E). Rectal temperature and rCBF at the ischemic parietal surface corresponding to the ischemic core were comparable among the four chimeric animal groups (Table 3). These findings suggested that BMDC-derived MMP-9 contributed to brain infarct size after ischemia/reperfusion. When the volume of brain swelling was subtracted from the total infarct size, the true tissue infarct size was attenuated only in KO/KO mice (Fig. 6F).

3. Discussion

We demonstrated the following two findings in this murine model of MCAO and reperfusion: (1) MMP-9 that is detectable in the ischemic brain is derived mainly from BMDC; (2) BMDC-derived MMP-9 contributes to BBB disruption at 1 h after reperfusion and infarct volume at 24 h after reperfusion.

3.1. MMP-9 in the ischemic brain is mainly derived from BMDC

The results from the experiments using bone marrow chimera indicate that BMDC including leukocytes are the main cellular source of MMP-9 in the ischemic brain, which is consistent with the previous findings (Gidday et al., 2005). The interpretation of the findings relies on the premise that WT/KO animals do not contain the MMP-9 gene in the brain parenchymal cells while KO/KO animals do not express MMP-9 in BMDC; including leukocytes. As argued by Gidday et al. (Gidday et al., 2005), transplanted bone marrow cells are unlikely to differentiate into brain parenchymal cells such as neurons, astroglia, and endothelial cells, particularly under the current experimental conditions: the bone marrow was transplanted 6 weeks before ischemia/reperfusion. Published studies using green fluorescent protein (GFP)-expressing bone marrow cells demonstrated GFP expression only in perivascular microglia but not in neurons and endothelial cells (Vallières and Sawchenko, 2003; Galimi et al., 2005). The very small number of the GFP-positive cells and their limited location including circumventricular organs and leptomeninges, both of which are outside of the ischemic area, hardly explain that these differentiated microglia are the primary source of such widespread MMP-9 activity that was detected in the ischemic brain in our current study (Fig. 5). Thus, it is highly likely that hematopoietic cells including leukocytes are the major source of MMP-9 in the ischemic brain, at least in WT/KO animals. Consistent with this possibility, we saw strong MMP-9 immunostaining in nucleated blood cells from WT/KO (Fig. 3C) and leukocyte-like cells in the ischemic brain from WT/KO mice (Fig. 5F).

We detected a moderate, but statistically detectable, reduction in infarct volume in KO/KO compared with WT/KO mice; however, the indirect infarct size corrected for brain swelling was not different between KO/KO and WT/KO animals (Fig. 6E and F). These findings suggest that parenchyma-derived MMP-9 could contribute to brain tissue injury although the amount of MMP-9 released from the parenchymal cells was below the level of detection. Nevertheless, both our current study and Gidday et al. (2005) showed that BMDC are the primary source of MMP-9 in the ischemic brain, and that wild-type bone marrow, regardless of the recipient genotype, is sufficient to produce brain tissue injury comparable to that in wild-type animals. In a mouse intracerebral hemorrhage model, there was a significant attenuation

in brain edema when recipient wild-type striatum was injected with blood from MMP-9 knockout mice (Tejima et al., 2007). Although this study did not determine whether MMP-9 was detectable in the brain of recipient animals and donor blood, the study showed the importance of MMP-9 genotype of circulating blood cells in the pathogenesis of cerebral edema after intracerebral hemorrhage.

Together with previously published studies, our current findings suggest that blocking MMP-9 during acute phase may be beneficial to treat ischemic stroke, although others have shown that MMP-9 inhibition extended to later phases at 7–14 days after ischemia is not functionally beneficial (Zhao et al., 2006). These authors demonstrated that the localization of MMP-9 shifts from the ischemic core to the periphery of the infarction during such a late recovery phase, providing evidence that MMP-9 may contribute to functional recovery in stroke penumbra. Whether BMDC are also the primary source of MMP-9 during such a recovery phase needs to be examined.

3.2. BMDC-derived MMP-9 and BBB disruption

The delivery of MMP-9 from the recruited leukocytes to the BBB seems to be locally and tightly controlled. Inhibition of the interactions between leukocyte and endothelial cell by anti-ICAM-1 antibody prevented MMP-9 expression in rat ischemic brain (Justicia et al., 2003), suggesting that the inter-cellular interactions between leukocytes and altered endothelial surface likely initiate the release of MMP-9 from leukocytes into the ischemic brain tissue. Our previous study using an *in vitro* flow-perfused model demonstrated that flow cessation/reperfusion significantly increased both luminal levels of MMP-9 and BBB permeability in the presence of circulating leukocytes (Krizanac-Bengez et al., 2006). Although it remains unknown whether MMP-9 is influential to the initial vascular changes after ischemia/reperfusion, our current study demonstrated that BMDC-derived MMP-9 contributes to the progression of BBB disruption (albumin and Evans blue extravasation).

In the current study, we found increases in gelatinolytic activity levels on the contralateral side at 1 h after reperfusion (Fig. 4B). These findings agree with the increased albumin and Evans blue leakage, compared to sham control, on the contralateral side at the same time point (Fig. 6B and D). As WT showed enhanced albumin leakage in the contralateral hemisphere compared with KO (Fig. 6C), these data suggest that BMDC-derived MMP-9 is influential to the contralateral BBB after ischemia/reperfusion. Post-stroke BBB dysfunction on the contralateral side has been under-recognized. Many previous studies regarding BBB disruption expressed data as a ratio to the presence of signal on the contralateral side, which does not adequately assess the changes on the contralateral side. Consistent with our findings, Belayev et al. (1996) reported that Evans blue leakage in the contralateral hemisphere was increased at 1–3 h after reperfusion following 2 h of MCAO in rats.

3.3. Neuronal association of MMP-9 activity

We observed that *in situ* DQ-gelatin cleaving activity was associated with neuronal nuclei (NeuN) (Fig. 5). Similar findings were also demonstrated by previously published studies using a mouse or rat MCAO model (Gu et al., 2005; Lu et al., 2008; McColl et al., 2008) and a rat cortical spreading depression study (Gursoy-Ozdemir et al., 2004). Since the *in situ* signal was not detectable in MMP-9 knockout mice, this signal was very likely an indicator of MMP-9 activity under our current experimental conditions. The neuronal localization of MMP-9 activity was unexpected since our current findings strongly suggested that neurons are not the main cellular source of MMP-9 in the ischemic brain. It is unknown whether MMP-9 released from BDMC is actively taken up by neurons or whether MMP-9 passively diffuses preferentially into injured neurons through the damaged plasmalemma. These issues may warrant further studies.

3.4. MMP-9 and reperfusion injury

Our current findings agree with the well-documented phenomenon that MMP-9 activity in the ischemic brain is enhanced by reperfusion (Lu et al., 2008; Gu et al., 2002). Since MMP-9 activity is regulated by its redox state (Gu et al., 2002), ischemia/reperfusion is able to enhance the activation of MMP-9. In addition, as suggested by the current study, re-circulation brings BMDC that are the main source of MMP-9 into the ischemic focus of the brain. Consistent with this notion, Gidday et al. (2005) demonstrated attenuated brain infarct size in MMP-9 knockout mice in a transient MCAO model but not in permanent MCAO models. Thus, the understanding of the mechanism of the MMP-9 release from BMDC may provide insights into a potential therapeutic target to block reperfusion-associated brain injury in ischemic stroke.

4. Materials and Methods

4.1. Animals

Male 129S6/SvEvTac mice (18–22 g) were purchased from Taconic (Germantown, NY). Monomeric red fluorescent protein-1 (mRFP1) expressing transgenic mice (Long et al., 2005) were purchased from The Jackson Laboratory (Bar Harbor, ME). A colony of mice lacking the gene for MMP-9 was maintained by cross breeding homozygous knockout mice. The breeding pairs of homozygous knockout mice (129/SV background) were obtained from Dr. Robert L. Fairchild at the Cleveland Clinic Lerner Research Institute who had received these mice as a gift from Dr. Robert M. Senior, Washington University School of Medicine, St. Louis, Missouri. (Vu et al., 1998). All procedures performed were reviewed and approved by the respective Institutional Animal Care and Use Committee of the Morehouse School of Medicine and the Cleveland Clinic.

4.2. Bone marrow chimeric mouse

The bone marrow chimeric animals were developed by use of whole body irradiation (9 Gy) with Cesium 137 γ -irradiation source (Sheperd or MDS Nordion) and bone marrow transplantation (3–5 million cells). The four types of chimeric mice were developed from combinations of MMP-9 knockout (KO) and wild-type (WT) mice as donors and recipients (Fig. 1). Additional type (RFP/KO) was developed from mRFP1 transgenic and KO mice as donors and recipients, respectively. The radiation dose was determined based on our preliminary findings that 9 Gy whole body irradiation was sufficient to abolish MMP-9 levels in the ischemic brain and spleen in SV129EV mice at 22 h after reperfusion following 2 h of MCAO (Fig. 2). During the first two weeks after irradiation, animals were treated with neomycin sulfate (0.2% w/v) in their drinking water. The neomycin treatment was tapered off subsequently, over a period of one week. Six weeks after bone marrow transplantation, the animals were subjected to 90 min of transient MCAO, described as follows.

4.3. Transient focal cerebral ischemia

MCAO was induced as previously described (Steele et al., 2008). Animals were anesthetized with 1.5% isoflurane in 68.5% N₂O and 30% O₂ throughout the procedures and for 10 min following reperfusion. Regional cerebral blood flow (rCBF) was monitored using a laser-Doppler flowmeter (FLO-C1, Omegawave, Tokyo, Japan) with a flexible probe affixed to the left skull (2 mm posterior to the bregma and 6 mm along the skull surface from and perpendicularly to the superior sagittal sinus). Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad. MCAO was induced on the left side with an 8-0 nylon monofilament coated with silicone and hardener mixture (Heraeus). A filament was then introduced into the left internal carotid artery through the external carotid artery and advanced until the tip occluded the anterior cerebral artery, and left in place for 90 min. For reperfusion, the filament was withdrawn and the carotid artery was re-opened.

4.4. Gel zymography

To determine the gelatinase levels, zymography was performed according to Zhang and Gottschall (1997) with modifications. Hemispheric and spleen tissues were homogenized in lysis buffer with a Potter glass homogenizer. The homogenates were centrifuged at $14,000 \times g$ for 15 min at 4°C. Supernatants were collected for analysis. The total protein concentrations were determined by Bradford assay. The supernatants were incubated for 1 h at 4°C with 50 μ l of gelatin-Sepharose 4B (GE Healthcare) to obtain gelatin binding fraction. The obtained fraction was used for standard gel zymography. The samples were electrophoretically separated in 8% polyacrylamide gels containing 0.1% porcine gelatin. The gel was incubated in renaturing buffer (2.5% Triton X-100 in distilled water) for 1 h, and then treated with developing buffer [0.05 M Tris-HCl (pH7.6), 0.15 M NaCl, 5 mM CaCl₂, 0.2% BRIJ-35] at 37°C for 20 min then for 24 h with refreshed developing buffer. Finally, the gel was stained with 0.5% Coomassie Blue R-250. The gel was scanned using an infrared imaging system (Odyssey, LI-COR Biosciences, Lincoln, Nebraska).

4.5. *In situ* detection of gelatinase activity

In situ zymography was performed to localize enzymatic activity on brain sections (Lee et al., 2004). Twenty μ m thick frozen coronal brain sections were obtained using a cryostat and were subsequently mounted onto glass slides. Sections were incubated with 0.05 M Tris-HCl (pH7.6), 0.15 M NaCl, 5 mM CaCl₂, containing 4 μ g/ml of FITC-conjugated DQ-gelatin (Invitrogen, Carlsbad, CA) over night at room temperature. For double staining with NeuN or MMP-9 immunostaining, sections were successively incubated with monoclonal anti-NeuN antibody (Chemicon, Temecula, CA) or rabbit anti-MMP-9 antibodies (Abcam, Cambridge, MA) at 4°C overnight followed by Alexa Fluor 568 goat anti-mouse IgG₁ secondary antibody (Invitrogen) or Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (Invitrogen) for 2 h at room temperature, respectively. Images were acquired with an epifluorescence microscope (Carl Zeiss Axioskop 2, Thornwood, NY) or a laser scanning confocal microscope system (Olympus IX71, Center Valley, PA).

4.6. Western blot analysis

MMP-9 protein level was determined by Western blot analysis using rabbit polyclonal MMP-9 antibodies (Abcam, Cambridge, MA). BBB leakage was measured by albumin extravasation into the brain tissue. Brain hemispheric tissue lysates prepared for zymography were used. The samples were separated by SDS-PAGE (4–20 % gradient) and transferred to a PVDF membrane. For albumin extravasation, immunoblotting was performed using goat anti-mouse albumin antibodies (Bethyl Laboratories, Montgomery, TX) and monoclonal anti- β actin antibody (Sigma-Aldrich, St. Louis, MO). Immunoblots were simultaneously incubated with IRDye 800CW anti-goat IgG and IRDye 680CW anti-mouse IgG (LI-COR) and scanned using the Odyssey infrared imaging system. The densitometric ratio (albumin/ β actin) was considered as albumin extravasation.

4.7. Determination of reconstitution of BMDC in chimeric mice

Complete blood count with differential was performed with Hemavet 950FS (Drew Scientific, Dallas, TX) at the Pathology Laboratory in Yerkes National Primate Research Center, Emory University (Atlanta, GA). The efficiency of reconstitution of BMDC in chimeric mice was assessed by MMP-9 immunocytochemistry of peripheral blood cells. Blood smear on a glass slide was fixed with 100% ethanol for 10 min and incubated with the MMP-9 antibodies (Abcam) followed by biotinylated goat anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA). Immune complex was treated with avidin-biotin-peroxidase solution (Vectastain ABC kit, Vector Laboratories) and visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO) with 0.003% H₂O₂ in 50 mM Tris-HCl, pH

7.5. The numbers of MMP-9 positive cells and total methyl green counterstained cells were counted in randomly selected fields under a microscope. At least 200 methyl green stained cells were counted in each blood sample. In addition, we counted under the epifluorescence microscope mRFP1 positive cells and total methyl green stained cells in the blood samples from RFP/KO mice.

4.8. Measurement of Evans blue extravasation

To measure BBB disruption at 1 h after reperfusion, 3% Evans blue (0.1 ml) was injected into the femoral vein 10 min before reperfusion following 90 min MCAO. At 1 hour after reperfusion, under 4% isoflurane anesthesia, blood was collected and perfusion with saline was made from the heart. Brains were immediately cut into 2 mm thick coronal sections and the dorsal planes of each section were scanned with the Odyssey infrared imaging system (LI-COR). The signals for 700 nm in each hemisphere were determined using the image analysis software installed in the system. Four numbers from the four sections were integrated for each hemisphere, and divided by blood concentration of Evans blue that was determined by the Odyssey infrared imaging system. The final number was considered as an indicator of BBB disruption and expressed as ratio to the mean value of non-ischemic sham-operated wild-type animals that received Evans blue injection after 80 min of 1% isoflurane and were euthanized after additional 70 min of isoflurane anesthesia.

4.9. Infarct size

Brain was quickly removed and frozen in 2-methylbutane chilled on dry ice. Twenty μ m thick coronal sections were made using the cryostat, which were subsequently mounted onto glass slides and stained with 0.5 % cresyl violet. Images of whole section were acquired using a 1.25X objective lens with a CCD camera. For each brain, infarct areal size in eight sections with 1 mm interval was determined with a computerized image analyzer (MCID, Imaging Research, Cambridge, UK) and infarct volume was calculated by summing the infarct area.

4.10. Statistical analysis

Data for blood count, rCBF and rectal temperature were expressed as mean $\pm$ SD and analyzed by one way analysis of variance (ANOVA) followed by Scheffe post-hoc analysis. Data for albumin extravasation, Evans blue extravasation, and infarct volume were shown as plots, and analyzed by Kruskal-Wallis test. Comparison between the two hemispheres was made by paired Student t test. Comparison between two animal groups was made by Mann-Whitney. $P < 0.05$ was considered statistically significant.

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Fig. 1.
Four types of chimeric mice after irradiation (9 Gy) and bone marrow transplantation with various combinations of wild-type (WT) and MMP-9 knockout (KO) as donor and recipient.

Fig. 2.

Zymograms showing gelatinase activity in brain (**A**), and spleen (**B**) from mice subjected to 2 h of MCAO followed by 22 h of reperfusion. Compared with irradiation to the head only, whole body irradiation markedly reduced MMP-9 activity in the spleen. Note remarkable reductions in MMP-9 activity levels in the brain and spleen from 9 Gy whole body irradiated mice. In contrast, the lower band presumably reflecting MMP-2 activity was not affected by irradiation. Mice were irradiated 7 days before brain ischemia/reperfusion. (**A**) Ischemic hemisphere from a non-irradiated mouse subjected only to 2 h MCAO with 22 h reperfusion was loaded on the second lane. C, contralateral hemisphere; I, ischemic hemisphere. **B**, Two cases are shown in each group.

Fig. 3. Immunocytochemical detection of MMP-9 in nucleated blood cells from chimeric mice. Nucleated blood cells were counter stained with methyl green. Blood cells from WT/KO (**C**) are MMP-9 positive as seen in WT (**A**) whereas blood cells from KO (**B**) and KO/WT (**D**) are negative for MMP-9 immunostaining. Note that, in addition to cellular staining, there is strong extracellular MMP-9 immunostaining surrounding methyl green stained cells (**A**, **C**). Bar = 20 µm.

Fig. 4.

Gel zymograms (A–C) and Western blot (D) showing MMP-9 in spleen and brain in mice that were subjected to 90 min MCAO followed by either 1 h (A and B) or 24 h (C and D) of reperfusion. Tissue homogenates were treated with gelatin Sepharose 4B and the isolated gelatinases were subjected to zymography or Western blot analysis. C, contralateral hemisphere; I, ischemic hemisphere; hMMP, human recombinant MMP; MW, molecular weight marker.

Fig. 5.

Fluorescent photomicrograph showing *in situ* gelatinase activity in four types of chimeric animal. **A and B**, Radiation and bone marrow transplantation from WT to MMP-9 KO mice (**B**) restored gelatinase activity (green) comparable to WT/WT (**A**) in the brain after 90 min MCAO with 24 h reperfusion. Double staining with NeuN immunostaining shows that gelatinase activity is primarily associated with neuronal nuclei (orange in the insets). *In situ* gelatinase activity was barely detectable in WT mice replaced with KO bone marrow (**C**) as seen in KO/KO (**D**). **E–G**, Confocal laser scanning image showing double labeling of *in situ* gelatinase activity (**E**) and MMP-9 immunostaining (**F**) in WT/KO. Gelatinase activity (green) was also associated with microvasculature (**E**) while MMP-9 immunostaining (red) was detected in leukocyte-like cells and microvasculature. **G**, There was association of gelatinase activity and MMP-9 immunostaining in microvasculature. Scale bar = 20 μ m.

Fig. 6.

MMP-9 from BMDC contributes to BBB dysfunction and infarct formation. Graphs showing relative protein levels of albumin in the brain (**A**, **B**, and **C**), Evans blue extravasation (**D**), and infarct size (**E**, **F**). The brains were collected from either naïve mice (**A**) or animals that were subjected to 90 min MCAO followed by 1 h (**B**, **D**), 18 h (**C**) or 24 h (**E**, **F**) of reperfusion. **A–C**, The ratio of Western blot density (albumin/ β -actin) was obtained from each brain hemisphere to measure BBB dysfunction. **D**, In separate animals, Evans blue extravasation corrected for Evans blue blood concentration was measured in each brain hemisphere to evaluate BBB dysfunction. Sham-operated non-ischemic animals injected with Evans blue were used as control. **E** and **F**, Infarct area was determined using eight cresyl violet stained coronal brain sections with intervals of 1 mm each and infarct volume was calculated and expressed as direct infarct size (**E**) or indirect infarct size corrected for brain swelling (**F**). Cont, contralateral hemisphere; Isch, ischemic hemisphere. Horizontal bars represent mean value. Statistical analysis was made by Mann-Whitney test (**A**) or Kruskal-Wallis test followed by Mann-Whitney test for the comparisons between two groups (**B–F**). **, $p < 0.01$ compared with the counterpart in WT (**C**) or WT/KO (**D**). The indicated numbers in (**E**) represent p value between two groups (Mann-Whitney test). #, $p < 0.05$ (Mann-Whitney test) compared with other three groups.

Table 1
Complete blood counting with differential

Animal group	WT (n = 5)	KO (n = 6)	WT/KO (n = 7)	KO/WT (n = 6)
Leukocytes				
WBC ($10^3/\mu\text{l}$)	7.1 ± 1.6	5.1 ± 1.3	4.6 ± 0.8	4.7 ± 0.5
Neutrophil ($10^3/\mu\text{l}$)	1.0 ± 0.3	1.0 ± 0.4	2.1 ± 0.3	1.5 ± 0.2
Lymphocyte ($10^3/\mu\text{l}$)	5.8 ± 1.2	3.8 ± 1.0	2.0 ± 0.5	2.6 ± 0.2
Monocyte ($10^3/\mu\text{l}$)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.3
Eosinophil ($10^3/\mu\text{l}$)	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.03
Basophil ($/\mu\text{l}$)	0	2 ± 4	4 ± 8	2 ± 4
Erythrocytes				
RBC ($10^6/\mu\text{l}$)	7.1 ± 1.0	7.0 ± 0.5	8.2 ± 1.6	8.2 ± 1.3
Hgb (g/dl)	10.9 ± 1.4	9.7 ± 0.9	11.7 ± 2.1	11.9 ± 1.9
Hct (%)	47.2 ± 7.8	41.2 ± 4.1	46.9 ± 11.6	52.9 ± 8.5
Thrombocytes				
PLT ($10^3/\mu\text{l}$)	304 ± 79	280 ± 61	355 ± 82	330 ± 43

WBC, white blood cells; RBC, red blood cells; Hgb, hemoglobin; Hct, hematocrit; PLT, platelets. Mean $\pm$ SD.

Table 2

WBC reconstitution

Animal group	WT (n = 5)	KO (n = 6)	WT/KO (n = 7)	KO/WT (n = 6)
MMP-9(+) WBC (%)	97.8 ± 1.6	0.8 ± 0.8	86.9 ± 2.5	10.5 ± 2.1

WBC, white blood cells. Mean ± SD.

Table 3
Rectal temperature (RT, °C) and regional cerebral blood flow (rCBF, % basal)

Animal group	Before	After MCAO (min)			After reperfusion
		30	60	80	
WT/WT (n=7)					
RT		37.2±0.1	37.0±0.2	37.1±0.2	37.2±0.2
rCBF	100	8.3±3.9	8.6±4.0	9.4±4.7	89.1±11.5
WT/KO (n=7)					
RT		36.8±0.3	37.3±0.5	37.2±0.3	37.1±0.2
rCBF	100	10.5±6.5	10.3±6.3	9.7±6.1	81.7±29.5
KO/WT (n=7)					
RT		36.8±0.4	37.1±0.3	36.8±0.5	37.0±0.3
rCBF	100	7.0±3.1	6.5±2.7	6.5±2.4	79.8±10.9
KO/KO (n=7)					
RT		36.9±0.2	37.3±0.3	37.2±0.2	37.0±0.3
rCBF	100	14.3±7.7	15.8±11.1	13.6±6.9	98.7±16.5

Mean ± SD.