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Matrix Metalloproteinase Inhibitors Attenuate Neuroinflammation Following Focal Cerebral Ischemia in Mice

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The aim of this study was to investigate whether matrix metalloproteinase (MMP) inhibitors attenuate neuroinflammation in an ischemic brain following photothrombotic cortical ischemia in mice. Male C57BL/6 mice were anesthetized, and Rose Bengal was systemically administered. Permanent focal ischemia was induced in the medial frontal and somatosensory cortices by irradiating the skull with cold white light. MMP inhibitors, such as doxycycline, minocycline, and batimastat, significantly reduced the cerebral infarct size, and the expressions of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and indoleamine 2,3-dioxygenase (IDO). However, they had no effect on the expressions of heme oxygenase-1 and neuroglobin in the ischemic cortex. These results suggest that MMP inhibitors attenuate ischemic brain injury by decreasing the expression levels of MCP-1, TNF-α, and IDO, thereby providing a therapeutic benefit against cerebral ischemia.

Key Words: Matrix metalloproteinase inhibitor, Monocyte chemotactic protein-1, Tumor necrosis factor-α, Indoleamine 2,3-dioxygenase, Photothrombotic cortical ischemia

INTRODUCTION

Ischemic stroke can elicit a neuroinflammatory reaction in the brain within a few hours after a stroke that lasts for days or weeks as a delayed tissue reaction to injury [1]. Several inflammatory mediators, such as chemokines, cytokines, adhesion molecules, and immune cells, contribute to acute cerebral ischemic injury [2].

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes that degrade the structural proteins in the extracellular matrix. Increased MMP activity is associated with the pathophysiology of various neurological diseases, including brain ischemia. In the central nervous system, excessive expression of MMPs contributes to pathological processes, including neuroinflammatory response in many neurological diseases [3], as well as to blood-brain barrier (BBB) injuries by degrading the neurovascular matrix [4]. Treatment with MMP inhibitors or MMP-neutralizing antibodies has been shown to decrease infarct volume and prevent BBB disruption after permanent and transient focal cerebral ischemia in rodents[5-7]. Although inhibition of MMPs has been reported to be associated with neuroprotective effects, the mechanism of protection in stroke is still unclear.

Monocyte chemoattractant protein-1 (MCP-1), a member of the CC chemokine family, is known to contribute to tissue damage via recruitment of inflammatory cells [8-10]. During ischemia, leukocyte infiltration is facilitated by the breakdown of the extracellular matrix because of altered extracellular MMP activity. MCP-1 activation is thought to be a crucial step in leukocyte infiltration, and MCP-1 expression following focal ischemia exacerbates ischemic damage [11].

Expression of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine considered the principal mediator of neuroinflammation, elicits a cascade of cellular events that culminates in neuronal death [12]. TNF-α is a potent stimulator of MMP expression and secretion in several types of cultured cells [13,14], and might be involved in BBB disruption and the initiation of inflammation in the brain [15,16].

Indoleamine 2,3-dioxygenase (IDO) is a heme-containing dioxygenase, which catalyzes the first and the rate-limiting step in the major pathway of L-tryptophan catabolism. IDO has been implicated in the pathogenesis of inflammatory neurologic diseases such as ischemic brain disease [17], poliovirus brain infection [18], acquired immunodeficiency dementia complex [19], and cerebral malaria [20]. Increase in the IDO level leads to neurological damage in case of global or focal brain ischemia [17,21]. Therefore, it is highly likely that the up-regulation of IDO expression in the brain results in the accumulation of quinolinic acid and neural degeneration.

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ABBREVIATIONS: MMP, matrix metalloproteinase; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α; IDO, indoleamine 2,3-dioxygenase; BBB, blood-brain barrier; HO, heme oxygenase; Ngb, neuroglobin.

Heme oxygenase (HO)-1 is an inducible form of HO, which is the rate-limiting enzyme in the degradation of heme to yield equimolar quantities of biliverdin, carbon monoxide, and iron [22,23]. A growing body of evidence suggests that HO may play an important role in protection against cerebral ischemia; therefore, induction of HO-1 protein expression following cerebral ischemia can lead to a beneficial outcome [24-26].

Neuroglobin (Ngb) is an oxygen-carrying vertebrate globin that is preferentially localized to cerebral neurons [27]. Overexpression of Ngb improves recovery from stroke in experimental animals [28,29]; thus. Ngb is thought to play a role in neuroprotection. Ngb offers a new target for protection against stroke or other ischemic insult to the brain.

In the present study, we speculated that development of progressive ischemic brain damage following acute cerebral ischemia implicates a crucial interaction between MMPs and neuroinflammation. We investigated whether the ability of MMP inhibitors to modulate the expression of neuroinflammatory mediators results in protection against permanent focal cerebral ischemia.

METHODS

Animals

The experimental protocols were in accordance with the Animal Care Guidelines of the Animal Experimental Committee of Pusan National University School of Medicine. Male C57BL/6 mice weighing 21～25 g were obtained from Koatech (Pyeongtaek, Gyeonggi-do, Korea). The mice were housed in a temperature-controlled room at 22～25°C. The mice were exposed to a 12-h light and dark cycle, with lights being switched on at 6:00 a.m. and were provided food and water *ad libitum*.

Photothrombotic cortical ischemia

Permanent focal ischemia was induced by cortical photothrombotic vascular occlusion according to the method of Schroeter et al. [30] with a slight modification as described previously [31]. The mice were anesthetized with chloral hydrate (450 mg/kg, i.p.), which allowed spontaneous respiration throughout the surgical procedure. The mice were placed in a head-holding adaptor (SG-4N, Narishige, Tokyo, Japan), and rectal temperature was monitored and maintained at 37±0.5°C with the help of a heating pad (Homeothermic blanket system; Harvard Apparatus Inc., Edenbridge, Kent, UK). A midline scalp incision and pericranial tissue dissection revealed the bregma and lambda points. A fiber optic bundle of a cold light source (KL 1500 LCD; Carl Zeiss, Göttingen, Germany) with a 4-mm aperture was centered using a micromanipulator at a lateral distance of 2 mm from the bregma. The aperture of the cold light source was placed as close as possible to the skull to avoid scattering of light, which can cause variability. The skull was then irradiated for 15 min starting at 5 min after the i.p. injection of Rose Bengal (0.1 ml of 10 mg/ml; Sigma-Aldrich, St. Louis, MO, USA). Then, the skin was sutured, and mice were allowed to awaken. The sham group was treated similarly, with the exception of irradiation and Rose Bengal injection. All mice tolerated the entire procedure well, showed no visible neurological or behavioral deficits, and survived brain ischemia.

Measurement of infarct size

The infarct size, including the infarct area and volume, was measured as described previously [31]. The mice were anesthetized with urethane (1 g/kg, i.p.) and killed by decapitation. The brain was quickly removed and chilled in ice-cold saline for 10 min. Five coronal sections (2-mm thick) were cut with a mouse brain matrix (RBM-2000C; ASI Instruments, Inc., Warren, MI, USA), beginning 2 mm posterior to the anterior pole, and the sections were immersed in a saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) at 37°C for 30 min and fixed by immersion in 10% neutral buffered formalin solution [32]. The posterior surface of each section was scanned using a digital camera, and the infarct area was quantified using an image analysis system (AxioVision LE; Carl Zeiss). The infarct volume for each section was then calculated by multiplying the infarct area by the section thickness. The total infarct volume in an animal was determined by summing up the infarct volumes of the 5 sections.

Immunohistochemistry

The animals were anesthetized and perfused transcardially with ice-cold 0.1 M PBS (pH 7.4) containing 20 U/ml heparin, followed by ice-cold 4% paraformaldehyde in PBS (pH 7.4). The brains were quickly removed, postfixed in 4% paraformaldehyde solution overnight at 4°C, and immersed in 20% sucrose until they sank. Coronal sections (7-μm thickness) were cut in a cryostat at −20°C by using a microtome (HM 560; Microm International GmbH, Waldorf, Germany). The sections were adhered to poly-L-lysine-coated slides, and allowed to dry at room temperature. After quenching endogenous peroxidase in 0.6% H_2O_2 and blocking non-specific protein-binding with CAS-Block (Zymed Laboratories Inc., South San Francisco, CA) for 30 min, the preparations were incubated overnight at 4°C with the primary antibodies, including monoclonal anti-rat MCP-1 antibody (1 : 300; ab8101, Abcam, Cambridge, MA, USA), monoclonal anti-mouse TNF-α antibody (1 : 200; sc-52746, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), polyclonal anti-mouse IDO antibody (1 : 200; ALX-210-432-C100, Alexis Biochemicals, San Diego, CA, USA), polyclonal rabbit anti-HO-1 (1 : 100; SPA-895, Stressgen Biotechnologies Co., Victoria, BC, Canada), and polyclonal anti-rabbit Ngb (FL-151) antibody (1 : 100; sc-30144, Santa Cruz). The sections were washed with PBS, and then incubated for 3 h with the appropriate secondary antibody, such as biotinylated anti-rabbit IgG, biotinylated anti-rat IgG, and biotinylated anti-mouse IgG, at a dilution of 1 : 100 (Vector Laboratories, Burlingame, CA, USA). After washing 3 times with PBS, the slides were incubated with avidin and biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) for 1 h. After washing in PBS, they were incubated with diaminobenzidine substrate kit (Vector Laboratories) and rinsed in water. The brain sections were examined for positive staining of MCP-1, IDO, HO-1, and Ngb by light microscopy.

Drugs

Doxycycline and minocycline (Sigma-Aldrich) were dissolved in saline, and batimastat (Tocris Bioscience, Bristol,

UK) was dissolved in 0.1% DMSO. Drugs were administered s.c. or i.p. in a volume of 1 ml/100 g.

Statistical analysis

The data are expressed as means±SEM. Statistical differences between groups were determined either using Student's *t*-test or by one-way analysis of variance, followed by Tukey's multiple comparison test as a post hoc test by using statistical software (Prism, version 5.01; GraphPad Software Inc., San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

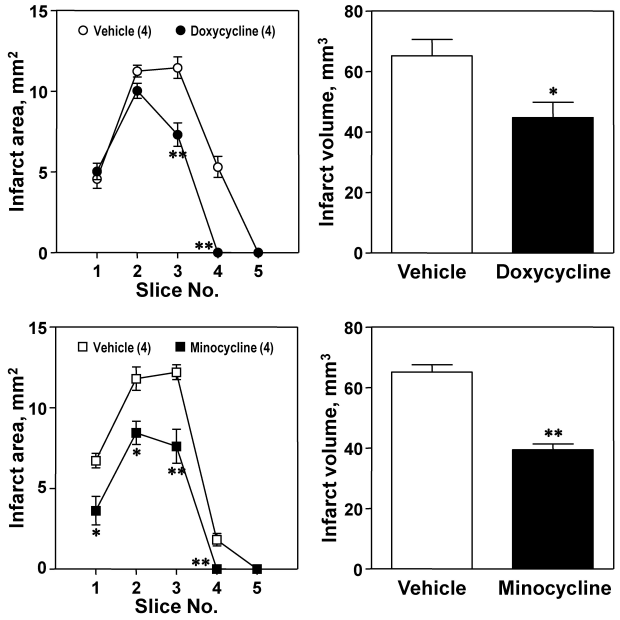

Fig. 1. Effects of doxycycline and minocycline on infarct area (left panels) and volume (right panels) in mice. Animals were treated with doxycycline (20 mg/kg, s.c.) or minocycline (45 mg/kg, s.c.) 30 min before and 2 h after ischemic insult, and were sacrificed 24 h after photothrombotic cortical ischemia. The numbers in parentheses indicate the numbers of animals. *$p<0.05$, **$p<0.01$ vs. vehicle group.

RESULTS

Infarct size

MMP inhibitors significantly reduced the infarct area and volume (Fig. 1). Doxycycline significantly reduced the infarct area in the 3rd and 4th sections compared with those in the vehicle group, leading to a significant reduction in infarct volume. Minocycline markedly reduced the infarct area in the 1st, 2nd, 3rd, and 4th sections compared with those in the vehicle group, leading to a marked reduction in infarct volume. The infarct size in cases treated with batimastat, showed a degree of reduction similar to that seen in cases treated with minocycline (data not shown).

Expression of MCP-1

MMP inhibitors significantly reduced the expression of MCP-1, which was overexpressed in the vehicle group (Fig. 2). Both doxycycline (40.5±0.5 positive cells/section) and minocycline (48.5±0.5 positive cells/section) significantly reduced the number of MCP-1 expressing cells in the ischemic cortex compared with that in the vehicle group (64.0±4.0 positive cells/section). Batimastat (12.5±4.5 positive cells/section) markedly reduced the expression of MCP-1 in the ischemic cortex compared with that in the vehicle group.

Expression of TNF-α

MMP inhibitors significantly reduced the expression of TNF-α, which was overexpressed in the vehicle group (Fig. 3). Doxycycline (35.0±3.0 positive cells/section) significantly reduced the expression of TNF-α in the ischemic cortex compared with that in the vehicle group (55.0±1.0 positive cells/section). Moreover, both minocycline (26.5±2.0 positive cells/section) and batimastat (18.5±2.5 positive cells/section) markedly reduced the expression of TNF-α in the ischemic cortex compared with that in the vehicle group.

Expression of IDO

MMP inhibitors significantly reduced the expression of IDO which was overexpressed in the vehicle group (Fig. 4).

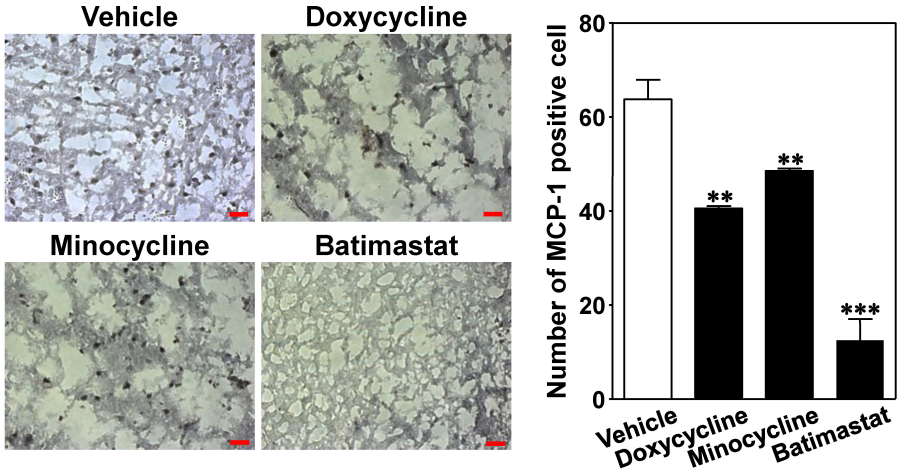

Fig. 2. Effect of matrix metalloproteinase (MMP) inhibitors on expression of monocyte chemotactic protein-1 (MCP-1) in ischemic cerebral cortex after photothrombotic cortical ischemia in mice. Doxycycline (20 mg/kg) or minocycline (45 mg/kg) was administered s.c. 30 min before and 2 h after ischemic insult, and batimastat (50 mg/kg) was administered i.p. 30 min before ischemic insult. The animals were sacrificed 24 h after photothrombotic cortical ischemia. Four animals were used in each group. Scale bar=50 μm. **$p<0.01$, ***$p<0.001$ vs. vehicle group.

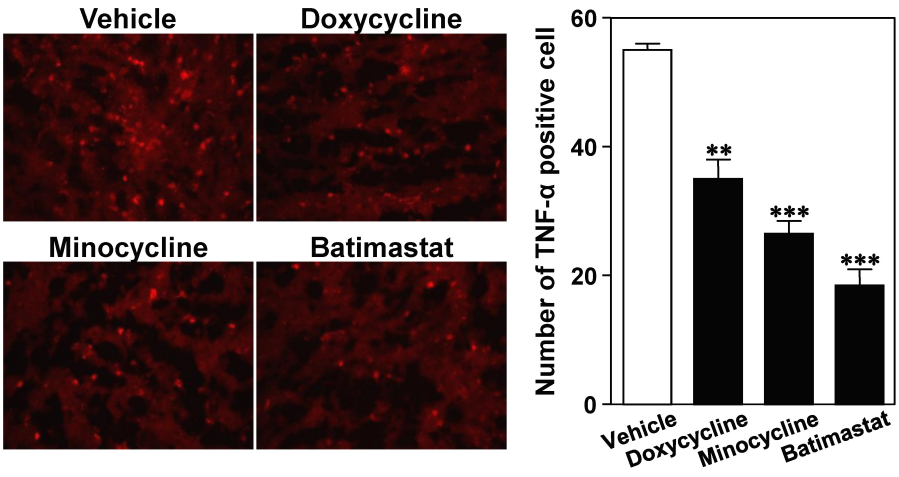

Fig. 3. Effect of MMP inhibitors on expression of tumor necrosis factor-α (TNF-α) in ischemic cerebral cortex after photothrombotic cortical ischemia in mice. Animals were treated with doxycycline (20 mg/kg) or minocycline (45 mg/kg) s.c. 30 min before and 2 h after ischemic insult, or with batimastat (50 mg/kg) i.p. 30 min before ischemic insult, and were sacrificed 24 h after photothrombotic cortical ischemia. Three animals were used in each group. Scale bar=50 μm. **p<0.01, ***p<0.001 vs. vehicle group.

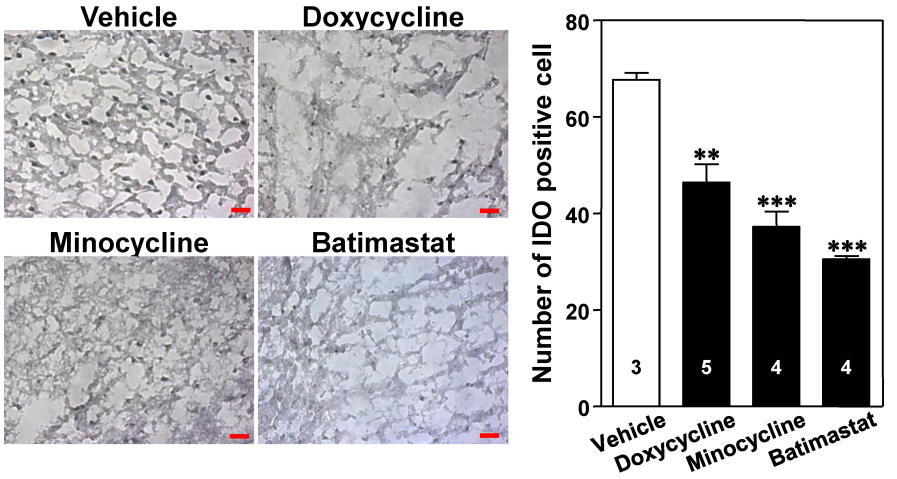

Fig. 4. Effect of MMP inhibitors on expression of indoleamine 2,3-dioxygenase (IDO) in ischemic cerebral cortex after photothrombotic cortical ischemia in mice. Animals were treated with doxycycline (20 mg/kg) or minocycline (45 mg/kg) s.c. 30 min before and 2 h after ischemic insult, or with batimastat (50 mg/kg) i.p. 30 min before ischemic insult, and were sacrificed 24 h after photothrombotic cortical ischemia. Numbers in columns indicate the numbers of animals. Scale bar=50 μm. **p<0.01, ***p<0.001 vs. vehicle group.

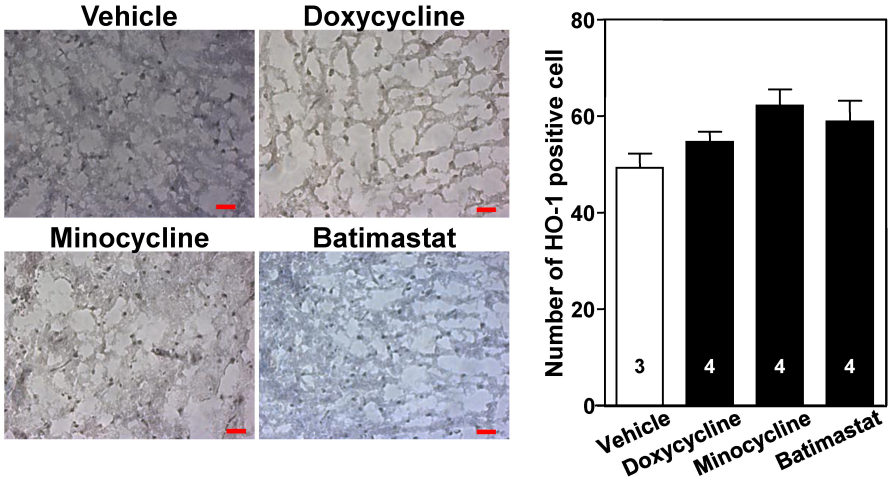

Fig. 5. Effect of MMP inhibitors on expression of heme oxygenase-1 (HO-1) in ischemic cerebral cortex after photothrombotic cortical ischemia in mice. Animals were treated with doxycycline (20 mg/kg) or minocycline (45 mg/kg) s.c. 30 min before and 2 h after ischemic insult, or with batimastat (50 mg/kg) i.p. 30 min before ischemic insult, and were sacrificed 24 h after photothrombotic cortical ischemia. Numbers in columns indicate the numbers of animals. Scale bar=50 μm.

The number of IDO-positive cells per section in the ischemic cortex was markedly reduced by treatment with not only doxycycline (46.4±3.8 positive cells/section) and minocycline (37.3±3.2 positive cells/section), but also batimastat (30.5±0.7 positive cells/section) as compared with those in the vehicle group (67.7±1.5 positive cells/section).

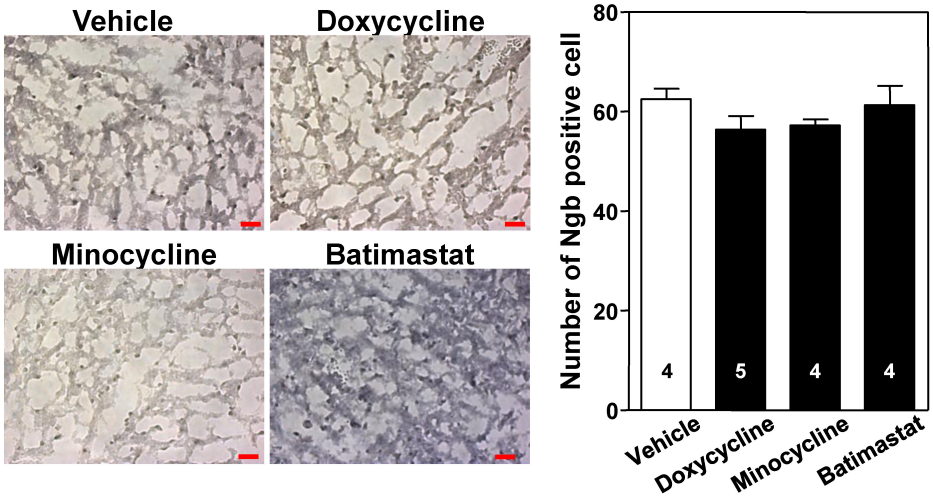

Fig. 6. Effect of MMP inhibitors on expression of neuroglobin (Ngb) in the ischemic cerebral cortex after photothrombotic cortical ischemia in mice. Animals were treated with doxycycline (20 mg/kg) or minocycline (45 mg/kg) s.c. 30 min before and 2 h after ischemic insult, or with batimastat (50 mg/kg) i.p. 30 min before ischemic insult, and were sacrificed 24 h after photothrombotic cortical ischemia. Numbers in columns indicate the numbers of animals. Scale bar=50 μm.

Expression of HO-1

HO-1 protein expression in the ischemic cortex markedly increased after photothrombotic cortical ischemia (49.3±2.9 positive cells/section). However, treatment with doxycycline, minocycline, or batimastat had little effect on the expression of HO-1, as shown in Fig. 5.

Expression of Ngb

Ngb protein expression in the ischemic cortex greatly increased after photothrombotic cortical ischemia (62.5±2.1 positive cells/section). However, neither treatment with tetracyclines (doxycycline or minocycline) nor treatment with batimastat affected the expression of Ngb (Fig. 6).

DISCUSSION

We examined the influence of MMP inhibitors on the recovery from brain tissue damage and neuroinflammation following photothrombotic cortical ischemia in mice. Pre- and postischemic treatments with MMP inhibitors significantly reduced the infarct size as well as the expression of neuroinflammatory mediators such as MCP-1, TNF-α, and IDO.

Focal cerebral ischemia induces microglial activation, astrocytosis, and leukocyte infiltration into the ischemic areas. These cells produce numerous growth factors, cytokines, and chemokines [33]. Neuroinflammation plays a pivotal role in the pathophysiology of cerebral ischemia [34], and inflammatory mediators such as cytokines, chemokines, adhesion molecules, and immune cells contribute to acute cerebral ischemic injury [2].

MMPs regulate extracellular matrix turnover and degrade the basal lamina [35-37]. The basal lamina plays a major role in maintaining BBB impermeability; therefore, cerebral ischemia leading to increased MMP expression and accelerated degradation of the endothelial basal lamina may cause disruption of the barrier [35,36]. Upregulated MMPs have been implicated in the propagation and regulation of the neuroinflammatory processes that accompany most central nervous system diseases [3,38].

A growing body of evidence indicates that abnormal MMP activity plays a role in the pathophysiology of cerebral ischemia. In particular, MMP-2 and MMP-9 are activated following focal brain ischemia and participate in the disruption of the BBB and hemorrhagic transformation following injury in animal models [5,7] and in stroke patients [39,40]. The resulting digestion of the endothelial basal lamina by MMP-2 and MMP-9 deregulates tight junctions, leading to the opening of the BBB. In cerebral ischemia-reperfusion, digestion of the endothelial basal lamina occurs as early as 2 h after ischemia [41], which may result in BBB permeability a few hours after ischemia [42].

MMP-9, which plays a pivotal role in the degradation of the BBB after focal cerebral ischemia [43], is expressed in human brain tissue after ischemic and hemorrhagic stroke [40]. The MMP-9 expression in the microvascular walls shows an early increase after cerebral ischemia, and selective inhibition of MMP-9 reduces the extent of brain injury after stroke [7]. Levels of MMP-9 peak at 48 h while those of MMP-2 peak at 5 days post-stroke. Astrocytic MMP-9 can mediate neurotoxicity *in vitro* [44], and neutrophil-derived MMP-9 contributes to the exacerbation of ischemic brain damage in mice with systemic inflammation [45].

Tetracyclines, including doxycycline and minocycline, are known to inhibit the levels and activities of various MMPs, including MMP-9 and MMP-2 [37,46], and are emerging as clinically usable non-specific MMP inhibitors. Batimastat (also known as BB-94), which belongs to a different class of MMP inhibitors, is a broad-spectrum MMP inhibitor that binds reversibly to the zinc-binding region of MMPs [47]. In the present study, doxycycline, minocycline, and batimastat, nonspecific and broad spectrum MMP inhibitors that are not restricted to a specific type of MMPs, were used to investigate the general anti-neuroinflammatory mechanism of MMP inhibitors. Treatment with these broad-spectrum MMP inhibitors significantly reduced the infarct size, showing a similar protective effect on permanent stroke as observed in previous reports [7,48].

Chemokines are regulatory polypeptides that mediate cellular communication and leukocyte recruitment in inflammatory and immune responses, and play an important role in the inflammatory response [49,50]. MCP-1 partic-

ipates in the inflammatory response and contributes to the development of ischemic brain injury [51]. In addition to its function in recruitment of inflammatory cells, MCP-1 also contributes to tissue damage via expression of MCP-1 mRNA in both astrocytes and microglia [9,10]. Recently, it was reported that the expression of MCP-1 in neurons increases significantly 12 h after focal brain ischemia, and increases in the levels of MCP-1 in astrocytes and microglia occur at later stages following the ischemic insult [33,52]. The infarct volume in mice deficient in MCP-1 was lower than that in focal brain ischemia [53]. Similarly, mice deficient in the gene for the MCP-1 receptor developed smaller infarcts, and lower levels of edema, leukocyte infiltration, and expression of inflammatory mediators [54]. In the present study, MMP inhibitors significantly reduced the expression of MCP-1 in the ischemic cortex. This finding is supported by the report that MMP is involved in the propagation and regulation of neuroinflammatory responses to ischemic brain injury [55].

Induction of TNF-α, which occurs following permanent middle cerebral artery occlusion, was reported to be associated with exacerbation of neurological deficits and infarct size, implicating this cytokine as a key player in ischemic brain injury [56]. At the same time, TNF-α affords neuroprotection in certain neurological conditions, including demyelination, neuronal excitotoxicity, and preconditioning with TNF-α [12]. In the present study, MMP inhibitors significantly reduced the expression of TNF-α in the ischemic cortex, resulting in a decrease in brain damage. Since broad-spectrum MMP inhibitors also inhibit the activity of metalloendopeptidases such as TNF-α converting enzyme, which cleaves membrane-bound pro-TNF-α to active soluble TNF-α [57,58], the effects of MMP inhibitors on TNF-α activity are not to be ignored. TNF-α contributes to the opening of the BBB by a mechanism involving soluble guanylyl cyclase and protein tyrosine kinase [59]. Therefore, it is necessary to determine whether the MMP inhibitors used in this study might have contributed to neuroprotection via reducing TNF-α activity.

The kynurenine pathway has been reported to be involved in several neurodegenerative diseases [60]. It is remarkable that an acute injury of the brain induces long-lasting alterations in tryptophan degradation with a shift toward detrimental metabolites [61]. IDO activity is highly induced under cerebral ischemia [17] by several cytokines including TNF-α, which is released from activated microglia [62,63]. Although the activation of glial cells was not examined in this study, it is assumed that resident brain cells such as microglia are rapidly activated following photothrombotic cerebral ischemia, as reported by Gehrmann et al. [64]. In the present study, it is of interest that the expression of IDO in the ischemic cortex was significantly reduced by MMP inhibitors. This finding strongly suggests that IDO plays a pathophysiological role in acute cerebral ischemia. Further investigations are needed to clarify the origin of IDO in the ischemic cortex.

HO-1, the inducible isoform of HO, catalyzes the rate-limiting step of heme oxidation to biliverdin, carbon monoxide, and free ferrous iron. Biliverdin is then rapidly converted by biliverdin reductase to bilirubin, a molecule with antioxidant properties, and free iron is sequestered by ferritin [65,66]. HO-1, a heat-shock protein (HSP-32), is a cytoprotective stress protein that is induced in the brain in response to permanent focal ischemia [24,25], and a hypoxia-inducible factor-1α-regulated gene [67]. The increased expression of HO-1 in ischemic brain tissue is probably of physiological consequence in the recovery of neuronal tissue following focal cerebral infarction, that leads to a beneficial outcome [26]. In the present study, MMP inhibitors had little effect on the expression of HO-1 protein, but nonetheless, it is feasible that MMP inhibitors might maintain and prolong the expression of HO-1 to protect against ischemic brain damage. Further investigation is needed to clarify the effect of MMP inhibitors on time-course expression of HO-1.

Ngb, an oxygen-carrying protein present mainly in neurons of the brain, offers a new target for protection against stroke or other ischemic insult to the brain. As an oxygen carrier and potential neuroprotective factor to neuronal cells, Ngb may enhance tolerance to ischemic insults [28,29]. Neuronal hypoxia and cerebral ischemia increase Ngb expression in cerebral neurons [8]. Ngb may help to promote neuronal survival from hypoxic-ischemic insults, since survival is reduced by inhibiting Ngb expression and enhanced by Ngb overexpression [28]. Ngb protects neurons from hypoxia in vitro [28], suggesting that this protein may have a role in sensing or responding to neuronal hypoxia, which could have implications for the pathophysiology and treatment of stroke [29]. In the present study, Ngb expression was significantly increased in the cerebral cortex 24 h after photothrombotic ischemic insults. Although all of the MMP inhibitors used in this study had little effect on the expression of Ngb, it is feasible that MMP inhibitors might maintain and prolong the expression of Ngb to protect against ischemic brain damage. Further investigation is needed to clarify the effect of MMP inhibitors on time-course expression of Ngb.

Taken together, it is suggested that MMP inhibitors, such as doxycycline, minocycline, and batimastat, reduce ischemic brain injury following photothrombotic cortical ischemia through inhibition of the expression of neuroinflammatory mediators, such as MCP-1 and IDO, and may thereby indicate new therapeutic strategies for cerebral ischemia.

ACKNOWLEDGEMENTS

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