Potential Contribution of Matrix Metalloproteinase-9 (MMP-9) to Cerebral Vasospasm After Experimental Subarachnoid Hemorrhage in Rats

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Abstract. This study investigated the possible involvement of matrix metalloproteinase 9 (MMP-9) in cerebral vasospasm (CVS) after subarachnoid hemorrhage (SAH) in rats. The CVS model was established by injection of fresh autologous nonheparinized arterial blood into the cisterna magna. Experiment 1 aimed to investigate the timecourse of the MMP-9 expression in the basilar artery after SAH. In Experiment 2, we chose the maximum time point of vasospasm (Day 3) and assessed the effect of SB-3CT (a selective MMP-9 inhibitor) on the regulation of cerebral vasospasm. The cross-sectional area of basilar artery was measured by H&E staining and the MMP-9 expression was assessed by immunohistochemistry analysis. The elevated expression of MMP-9 was detected in the basilar artery after SAH and peaked on day 3. After intracisternal administration of SB-3CT, the vasospasm was markedly attenuated after blood injection on day 3. Our results suggest that MMP-9 is increasingly expressed in a parallel time course to the development of cerebral vasospasm in this rat experimental model of SAH and that the administration of the specific MMP-9 inhibitor could prevent or reduce cerebral vasospasm caused by SAH.

Key words: MMP-9; Subarachnoid hemorrhage; Vasospasm

Introduction

Cerebral vasospasm is the most common cause of disability and death in patients suffering from aneurysmal subarachnoid hemorrhage (SAH) [1-3]. Treatment of cerebral vasospasm is considered a major goal in the management of patients surviving SAH. However, the exact molecular mechanism of cerebral vasospasm still remains obscure, hindering the development of effective and specific treatment paradigms [2]. Possible mechanisms may include: up-regulated vascular inflammation, endothelial dysfunction, increased amounts of free radical products and oxidative stress, adenosine diphosphate (ADP)-induced vasomotor changes, inhibition of the nitric oxide (NO) pathway or the protein kinase C (PKC) pathway in vascular smooth muscle, in which inflammatory response and endothelial injury play important roles in the pathological process of vasospasm [2].

Previous studies have demonstrated that matrix metalloproteinase 9 (MMP-9) plays an important role in the pathogenesis of cerebral inflammation and endothelial dysfunction [4-5]. As mentioned by Rosenberg et al [5], MMP-9 production is up-regulated in LPS-injured brain tissue and is instrumental in regulating the size-differentiated opening of the blood-brain barrier during acute neuroinflammation. At the same time, Higashida reported that concurrently elevated expression of MMP-9 in traumatically injured brain tissues temporally coincides with brain edema formation and

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BBB disruption, which was reduced by selective inhibition of MMP-9 [6]. However until now, no study investigating the role of MMP-9 in cerebral vasospasm has been found in the literature. The aim of the current study was to evaluate the basilar arterial MMP-9 change following SAH and determine the potential role of MMP-9 in the development of cerebral vasospasm. We hypothesized that inhibiting MMP-9 might attenuate the development of cerebral vasospasm in this rat SAH model.

Materials and Methods

Animals. The animal use and care protocols, including all operation procedures, were approved by the Animal Care and Use Committee of Soochow University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institute of Health. Forty-eight male Sprague-Dawley rats weighing from 350 to 400 g were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). They were acclimated in a humidified room and maintained on the standard pellet diet at the Animal Center of Soochow University for 10 days before the experiment. The temperature in both the feeding room and the operation room was maintained at about 25 °C.

Rat SAH Model. Male Sprague-Dawley rats (350 to 400 g) were anesthetized on day 0 with pentobarbital (40 mg/kg, IP) and allowed to breathe spontaneously. With the aid of a surgical microscope, a small suboccipital incision was made, exposing the arch of the atlas, the occipital bone, and the atlanto-occipital membrane. With a 27-gauge needle, the atlanto-occipital membrane was tapped carefully into the cisterna magna. Freshly autologous nonheparinized blood (0.3 ml) from the femoral artery was injected during a period exceeding 2 minutes. Immediately after the injection of blood, the hole was sealed with absorbable sponge to prevent fistula, and the wound was sutured. The rats were then placed in a head-down prone position at a 30-degree angle for 30 minutes to hold the blood in the basal cisterns. The animal recovered from the effects of anesthesia and was returned to its cage. In control animals, the same technique was applied to the injection of sterile saline instead of blood.

Experimental design. In experiment 1, thirty-six rats were assigned randomly to 6 groups: Control group, SAH Day 1, Day 3, Day 5, Day 7, and Day 14 groups. The animals in Day 1, Day 3, Day 5, Day 7, and Day 14 groups were subjected to experimental SAH on days 0 and were killed on days 1, 3, 5, 7, and 14, respectively (n=6 for each group). The groups in Experiment 2 consisted of the vehicle-treated group (n=6) and the SB-3CT treated group (n=6).

SB-3CT (a selective MMP-9 inhibitor) was purchased from Sigma-Aldrich Corporation, USA.
Because of the prohibitive costs of administering SB-3CT systemically, in this study we used intracisternal injections to limit the amount of SB-3CT. SB-3CT was reconstituted with normal saline (NS) with doses of 0.3 mg per animal and the proportion was 0.3 mg (SB-3CT):0.2 ml (NS). In the animals of the SB-3CT treated group, SB-3CT was injected into the cisterna magna once as the blood injection manner 30 minutes before blood injection. The animals of the vehicle-treated group received equal volumes (0.2 ml) of NS at the corresponding time point. All the rats in Experiment 2 were killed on day 3, which was the time point of the highest MMP-9 expression and vasospasm according to the result of Experiment 1.

**Perfusion-fixation.** The rats scheduled for death were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (0.5 mg/kg). Perfusion-fixation was then performed. The thorax was opened with a cannula placed in the left ventricle, the descending thoracic aorta clamped, and the right atrium open. Perfusion was begun with 250 ml of physiological phosphate buffer solution (PBS, pH 7.4) at 37 °C, followed by 250 ml of 10% buffered formaldehyde under a perfusion pressure of 120 cm H$_2$O. After perfusion-fixation, the whole brain was removed and immersed in the same fixative solution.

**Measurement of Blood Vessel Cross-Sectional Area.** The degree of cerebral vasospasm was evaluated by the measurement of the basilar artery lumen’s cross-sectional area. The formalin-fixed and paraffin-embedded basilar artery sections (4 mm in thickness) were deparaffinized, hydrated, washed, and stained with hematoxylin and eosin. Micrographs of the basilar arteries were then put into the computer. An investigator without knowledge of the group setting determined the cross-sectional areas and wall thicknesses of blood vessels using the High Definition Medical Image Analysis Program (HMIAP-2000, developed by Tongji Medical University, China). The areas were calculated by measuring the perimeter of the actual vessel lumen and then calculating the area of an equivalent circle (area=$\pi r^2$, where $r$ = radius) based on the calculated equivalent $r$ value from the perimeter measurement ($r$ = perimeter/$2\pi$), thus correcting for vessel deformation and off-transverse sections. For each vessel, three sequential sections (midpoint of the proximal, the middle, and the distal) were taken, measured, and averaged.

**Immunohistochemical study.**

Immunohistochemistry on formalin-fixed paraffin-embedded sections was performed to determine the immunoreactivity of MMP-9. Sections were
Contribution of Matrix Metalloproteinase-9 to Cerebral Vasospasm
deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ for 5 minutes, followed by a brief rinse in distilled water and a 15-minute wash in PBS. Sections were placed in 10 mmol/L citrate buffer (pH 6.0) and heated in microwave oven at 95°C for 30 minutes. Sections were cooled at room temperature for 20 minutes and rinsed in PBS. Non-specific protein binding was blocked by a 40-minute incubation in 5% horse serum. Sections were incubated with primary antibody (anti-MMP-9 from Santa Cruz Biotechnology, Inc., California, USA) for 1 hour at room temperature, followed by a 15-minute wash in PBS. Sections were incubated with horseradish peroxidase (HRP)-conjugated IgG (1:500 dilution, Santa Cruz Biotechnology, Inc., California, USA) for 60 minutes at room temperature. DAB was used as chromogen, and counterstaining was done with hematoxylin. Sections incubated in the absence of primary antibody were used as negative controls. Microscopy of the immunohistochemically stained tissue sections was performed by an experienced pathologist blinded to the experimental condition. The evaluation of sections was therefore undertaken by assessing the intensity of staining (5 grades). “0” indicates no positive cell; “1” indicates very low density of positive cells; “2” indicates a moderate density of positive cells; “3” indicates a higher, but not maximal, density of positive cells; and “4” indicates the highest density of positive cells.

**Statistical Analysis.** All data were presented as mean ± SD. SPSS 12.0 was used for statistical analysis of the data. All data were subjected to Student’s t test or one-way ANOVA. Differences between experimental groups were determined by the Fisher’s LSD post-test. Statistical significance was inferred at P<0.05.

**Results**

**General observation.** No significant changes in body weight mean arterial blood pressure, temperature, or injected arterial blood gas data were detected in any of the experimental groups (data not shown). As shown in **Figure 1**, the rats in the Day 1 and Day 3 groups exhibited blood clots over the
basal surface of the brainstem, but the blood staining at the base of the brainstem was not observed in the Day 5, Day 7, or Day 14 groups.

The basilar artery cross-sectional area. There was a significant difference in the basilar artery cross-sectional area among groups (P<0.001, **Figure 2G**). Between the Day 3 group (22967.3±7671.4 μm²) and the control group (58818.5±2532.1 μm²), there are extremely significant differences in the basilar artery cross-sectional areas (P<0.01, **Figures 2A, 2C, and 2G**). And so it is between the Day 1, 5, and 7 groups (43178.4±6329.1, 33670.2±6713.4, and 49423.2±4116.3 μm², respectively) and the control group (P<0.05, **Figures 2A, 2B, 2D, 2E and 2G**). No significant difference was detected between the Day 14 group (57425.1±6017.3 μm²) and the control group (P>0.05, **Figures 2F and 2G**).

**Immunohistochemistry for MMP-9 expression.**

To assess the localization of MMP-9 expression, immunohistochemistry for MMP-9 was performed. A few MMP-9-positive cells were observed in the control group, which indicates the constitutional expression of MMP-9 in the normal basilar arteries of rats (**Figure 3A**). Increased MMP-9-positive cells in the SAH groups could be found in the basilar artery (**Figures 3B and 3C**). On days 1 and 3, MMP-9 immunoreactivity was mainly present in the endothelial cells but was also present in the smooth muscle cells (**Figures 3B and 3C**). Quantitative analysis showed a low MMP-9 immunoreactivity in the control group, with an average score of 0.43±0.12. In contrast, MMP-9 immunoreactivity was increased in the Day 1, 3, 5 and 7 groups, with average scores of 2.10±0.12, 3.77±0.12, 2.43±0.12, and 1.77±0.12. Significant differences were found both between the Day 1, 3, 5 or 7 groups, and the control group (P<0.05).

**Effect of SB-3CT on MMP-9 expression in SAH basilar arteries.** To determine the influence of SB-3CT on MMP-9 expression in the basilar arteries post SAH, immunohistochemistry was performed (as described in Materials and Methods) to detect changes in MMP-9 immunoreactivity. **Figure 4** showed a high level of MMP-9 in the vehicle-treated SAH group. On day 3 after SAH, the up-regulated levels of MMP-9 were significantly decreased in the SB-3CT treated group (P <0.01) (**Figure 4**).

**Impact of SB-3CT administration on cerebral vasospasm after SAH.** As shown in **Figure 5**, there was a significant difference in the cross-sectional areas of basilar artery of the vehicle-treated group (n=6) and of the SB-3CT treated group (n=6) on day 3 following SAH (P< 0.01). A significant difference (P < 0.01) was detected between the SB-3CT group (32361.3 ± 6265.4 μm²) and the vehicle-treated group (22336.2 ± 5211.3 μm²) (**Figure 4**).

**Discussion**

Delayed or chronic vasospasm is the leading cause of morbidity and mortality after aneurismal SAH.
Contribution of Matrix Metalloproteinase-9 to Cerebral Vasospasm

Although cerebral vasospasm after SAH has been the subject of substantial research interest, the underlying pathogenic mechanisms remain obscure. The main findings of this study are as follows: 1) MMP-9 was up-regulated in the basilar arterial wall during cerebral vasospasm after SAH in rats; 2) the time-course study showed that the MMP-9 expression peaked on day 3 and recovered on day 14 following SAH; 3) SAH-induced increases of MMP-9 expression could be suppressed by intracisternal administration of SB-3CT; and 4) SB-3CT could reduce the degree of vasospasm of the basilar arteries. These findings suggest for the first time that up-regulation of MMP-9 may potentially play a role in aggravating the development of cerebral vasospasm in the rat SAH model.

A growing body of evidence supports the critical role of MMP-9 in the pathophysiology of SAH [8-11]. As mentioned by Guo [8-9], MMP-9 was activated in the cortex at the early phase after SAH in rats. Activation of MMP-9 resulted in the degradation of laminin, which may contribute to neuronal cell death (apoptosis) and brain edema. SB-3CT, the inhibitor of MMP-9, decreased the apoptosis of neurons, reduced brain edema, and improved the behavior and activities of rats. Another previous study demonstrated that serum MMP-9 concentration can effectively predict the onset of delayed cerebral vasospasm before the onset of Transcranial Doppler (TCD) velocity changes or neurological deterioration. The likelihood of delayed cerebral vasospasm increased approximately 20-fold with a large elevation in the level of MMP-9, which predicted the onset of delayed cerebral vasospasm with up to 92% accuracy [10].

However until now, no data about MMP-9 and vasospasm after SAH was found in the literature. In our current research, we first reported the up-regulation of MMP-9 in the basilar arteries following SAH. The MMP-9 activity was time-dependent; the maximal activation occurred on day 3 post-SAH. The elevated MMP-9 in the arterial wall suggested that MMP-9 expression and signaling could participate in the pathogenesis of cerebral vasospasm induced by SAH. Our data also demonstrated that treatment with SB-3CT reduced the vascular expression of MMP-9 and resulted in a significant decrease of the degree of vasospasm. The medial layer of smooth muscle cells presented positive MMP-9 expression a few days after SAH, which may indicate that the resident cells in the medial layer should be targets for the immunological response.

As mentioned in the introduction section, inflammation has increasingly been proved to be involved in the development and maintenance of cerebral vasospasm [12]. Previous studies have described inflammatory processes in SAH, including subarachnoid and perivascular leukocytic recruitment, infiltration, and activation, cytokine production, immunoglobulin and complement activation, and transcription factor activation [13]. A certain amount of the animal reports have also been directed towards some more specific endpoints related to inflammatory reactions. Furthermore, a large number of tests on the capacity of anti-inflammatory drugs to prevent vasospasm have been

Figure 5. Upper: (A) The vehicle-treated group (n=6) and (B) SB-3CT treated group (n=6). Bottom: Histogram of the average cross-sectional area of the basilar arteries from both groups. There is a significant difference in the basilar artery cross-sectional areas of the SB-3CT group and the vehicle-treated group (P<0.01). Results are represented as means ± SD of six rats in each group. ## P <0.01 versus vehicle group.
performed [12]. In the previous research regarding MMP-9 and neuroinflammation, Mun-Bryce reported the up-regulation of MMP-9 protein during inflammation in the brain. At the same time, the authors found that the decrease in blood-brain barrier permeability was linked to a fall in MMP-9 production in the animals treated with MMP-9 inhibitor, suggesting a possible therapeutic intervention against the potentially damaging processes of neuroinflammation [5]. We tentatively propose that the therapeutic benefit of SB-3CT administration might result from its salutary effect on modulating neurovascular inflammatory response by down-regulating MMP-9 expression.

This preliminary study did not determine the bioavailability of SB-3CT. In addition, we tried only one dosage at the early stage of experimental SAH, which could repress vascular MMP-9 expression significantly, as confirmed by the following immunohistochemical studies. It is conceivable that MMP-9 mediated vascular inflammation and basement membrane’s dysfunction are important steps in the initiation of vasospasm, but its detailed molecular mechanisms and the optimum duration of SB-3CT treatment call for further research. Our data provide the first evidence for the possible role of MMP-9 in the pathogenesis of cerebral vasospasm. Although these findings are preliminary, they provide encouragement that inhibitors of cellular inflammation may prove a novel clinical avenue for the treatment of cerebral vasospasm.

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