Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in early focal cerebral infarction following urokinase thrombolysis in rats

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Abstract
Activity of matrix metalloproteinase-9 increases following cerebral ischemia/reperfusion, and is associated with cerebral microvascular permeability, blood-brain barrier destruction, inflammatory cell infiltration and brain edema. Matrix metalloproteinase-9 also likely participates in thrombolysis. A rat model of middle cerebral artery infarction was established by injecting autologous blood clots into the internal carotid artery. At 3 hours following model induction, urokinase was injected into the caudal vein. Decreased neurological severity score, reduced infarct volume, and increased expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 were observed in the cerebral cortex 24 hours after urokinase thrombolysis. These results suggest that urokinase can suppress damage in the acute-early stage of cerebral infarction.

Key Words
cerebral infarction; urokinase; thrombolysis; matrix metalloproteinase-9; tissue inhibitor of metalloproteinase-1; neural regeneration

Abbreviations
MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; NSS, neurological severity score; TTC, 2,3,5-triphenyltetrazolium chloride

INTRODUCTION
The optimal therapeutic measure for acute cerebral infarction is to recanalize occluded cerebral vessels, and to recover blood supply before irreversible ischemic injury occurs to brain tissues[1-2]. Thrombolytic therapy is an effective method for acute ischemic stroke, but this method can increase the risk of hemorrhagic transformation following ischemia[3-4]. Thrombolysis-associated hemorrhagic transformation is associated with free radical release, increased activities of neutrophils and macrophages, and protease release following ischemia/reperfusion[5-6]. Matrix metalloproteinase-9 (MMP-9) is mainly synthesized and secreted by neutrophils, monocytes, vascular endothelial cells, smooth muscle cells, astrocytes, microglia and macrophages[7-9]. MMP-9 activation is mediated by proteins that activate the proenzyme and is negatively regulated by inhibitors[10]. MMP-9 activity increases following cerebral ischemia/reperfusion, and its expression is strongly linked to cerebral microvascular permeability, blood-brain barrier destruction, inflammatory cell infiltration and brain edema[10-11], suggesting

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that it plays an important role in cerebral ischemia/reperfusion by degrading collagen, laminin and fibronectin, which are the main components of the perivascular basement membrane. In this study, we investigated changes in MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression, and their effects on thrombolysis after urokinase administration in rats with focal cerebral infarction.

RESULTS

Quantitative analysis of experimental animals
A total of 100 rats were included in this study, of which 10 were included in the sham-surgery group, and the remaining 90 participated in the middle cerebral artery infarction model of ischemic injury. Of these, 80 rats underwent successful middle cerebral artery infarction, and were randomly assigned to model and urokinase groups. At 3 hours following blood clot injection, saline or urokinase was injected into the caudal vein of rats in the model and urokinase groups, respectively.

Sham-operated rats were only used for determining infarct volume.

Urokinase thrombolysis lowered the neurological severity score (NSS) in rats with cerebral infarction
NSS scores did not change at 24 hours following saline administration in rats in the model group ($P > 0.05$). NSS scores were significantly reduced in the urokinase group at 24 hours following urokinase administration ($P < 0.01$; Table 1).

Table 1 Neurological severity score (NSS) scores before and after thrombolysis in rats with cerebral infarction

| Time              | Model group | Urokinase group |
|-------------------|-------------|-----------------|
| Pre-administration| 9.28±1.14   | 9.09±1.33       |
| 24 hours post-administration | 9.19±0.93 | 6.38±1.11       |
| $t$                | 0.27        | 7.00            |
| $P$                | 0.79        | 0.00            |

The NSS score ranges from 0 to 14. The higher the NSS score, the poorer the neurological function. The data are expressed as mean ± SD. A total of 40 rats from each group were included. Differences were compared using two-sample $t$-test at various time points.

Urokinase thrombolysis reduced infarct volume in rats with cerebral infarction
2,3,5-triphenyltetrazolium chloride (TTC) staining demonstrated that the infarcted region comprised the area supplied by the left middle cerebral artery, primarily the cortex, but also the basal ganglia and hippocampus. At 24 hours following thrombolysis, infarct volume in rats in the urokinase group ($59.24 ± 8.25$ mm$^3$) was significantly smaller than in the model group ($94.90 ± 11.09$ mm$^3$, $t = 13.494$, $P = 0.00$; Figure 1).

Urokinase thrombolysis increased MMP-9 and TIMP-1 expression in rats with cerebral infarction
Immunohistochemical staining revealed MMP-9 and TIMP-1 expression in the cytoplasm of cells surrounding the infarct region in the model and urokinase groups. MMP-9 and TIMP-1 expression in the rat cortex was significantly greater in the urokinase group than in the model group ($P < 0.01$; Figure 2, Table 2).

Table 2 Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in the cortex of rats with cerebral infarction

| Model group | Urokinase group |
|-------------|-----------------|
| MMP-9      |                 |
| TIMP-1     |                 |

Figure 1 Infarct volume at 24 hours following thrombolysis (2,3,5-triphenyltetrazolium chloride staining). Unaffected brain regions are stained red, and infarcted regions are unstained. (A) Sham-surgery group; (B) model group; (C) urokinase group.

Figure 2 Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in the cortex of rats with cerebral infarction (immunohistochemical staining, × 200). MMP-9 and TIMP-1 expression in the rat cortex was significantly greater in the urokinase group than in the model group. Arrows indicate positive expression.
Urokinase thrombolysis increased MMP-9 and TIMP-1 mRNA expression in rats with cerebral infarction

In situ hybridization showed MMP-9 and TIMP-1 mRNA expression in the cytoplasm of cells surrounding the infarct region in the model and urokinase groups. MMP-9 and TIMP-1 mRNA expression in the rat cortex was significantly greater in the urokinase group than in the model group ($P < 0.01$; Figure 3, Table 3).

**DISCUSSION**

Blood-brain barrier disruption is the pathophysiological basis for the hemorrhagic transformation of cerebral infarction. MMP-9 has been shown to play a critical role in reperfusion-induced blood vessel destruction. A previous study showed that the MMP inhibitor BB-94 can decrease the risk of secondary hemorrhage in rats with cerebral infarction\(^\text{[12]}\). A monoclonal antibody to MMP-9 noticeably diminished infarct volume in a rat model of local ischemia\(^\text{[13-15]}\). TIMP therapy decreased vasogenic brain edema and infarct volume\(^\text{[16-17]}\). In this study, NSS scores did not change 24 hours following saline injection, but were significantly reduced 24 hours following urokinase thrombolysis. Moreover, infarct volume was decreased following urokinase thrombolysis. These results suggest that urokinase thrombolysis has positive effects on cerebral infarction in the acute-early stage, which is consistent with previous results\(^\text{[18-19]}\). In the present study, immunohistochemistry and in situ hybridization demonstrated that MMP-9 protein and mRNA levels were significantly increased following urokinase thrombolysis in rats with cerebral infarction. This enhanced expression might be induced by ischemia/reperfusion injury, or it may be due to urokinase, as this enzyme can enhance MMP-9 expression in the ischemic region, as shown by a previous study\(^\text{[19]}\). These observations indicate that MMP-9 plays an important role in secondary brain injury following thrombolysis. Our present study also demonstrated that TIMP-1 protein and mRNA expression was greater in the urokinase group than in the model group, indicating upregulation of endogenous TIMP expression after thrombolysis, which might be a protective reaction secondary to MMP-9 upregulation.

In summary, urokinase thrombolysis for 24 hours increases MMP-9 and TIMP-1 expression in the cerebral cortex of rats with cerebral infarction, improving neurological function. This demonstrates that urokinase...
thrombolysis has therapeutic effects on cerebral infarction in the acute-early stage.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
Experiments were performed at the Laboratory of the Institute of Cerebrovascular Disease, Medical College, Qingdao University, China, from January 2008 to June 2009.

**Materials**
A total of 100 clean healthy male Wistar rats, aged 10 weeks old and weighing 280–320 g, were purchased from the Experimental Animal Center, Medical College, Shandong University, China (license No. SCXK (Lu) 20080006). The rats were housed at 21–27°C with a humidity of 45–55%. Protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[20].

**Methods**

**Preparation of autologous thrombus**
Autologous thrombus was prepared in accordance with the method of Busch *et al*[21]. Briefly, rats were intraperitoneally anesthetized with 10% chloral hydrate. 1 mL of blood from the right common iliac vein was placed in a No. 22 indwelling needle for 2 hours at room temperature. A strip of thrombus (0.5 mm diameter) was obtained, washed in saline, stored in a water bath at 37 °C for 24 hours, and then trimmed into 0.5-mm-diameter particles.

**Establishment of cerebral infarction model**
The rats were intraperitoneally anesthetized with 10% chloral hydrate, 350 mg/kg. A medium incision was made on the neck. The left common carotid, external carotid and the internal carotid arteries were isolated under a microscope (Olympus, Tokyo, Japan). After thermal coagulation, the external carotid artery was cut, and the trunk of the artery was ligated and dissociated. The common carotid and internal carotid arteries were temporarily blocked. The external carotid artery stump was dragged slightly, and a small opening was made in the external carotid artery. The No. 24 indwelling needle was inserted, and the core was pulled out. Six thrombus particles were injected into the internal carotid artery, passing through the common carotid artery bifurcation point and the internal carotid artery, traversing into the anterior cerebral artery. The blood supply of the left middle cerebral artery was blocked. The external carotid artery stump was ligated, and blood flow to the common carotid artery was restored[22-23]. Rectal temperature was maintained at 37 ± 0.5°C. After the rats regained consciousness, NSS testing was performed in accordance with a previous study[24]. NSS scores > 7 represent successful model establishment[25-26]. Rats with successful cerebral infarction were characterized by Homer’s syndrome on the left, weak or absent corneal and panic reflexes, right limb paralysis, difficult crawling (not straight), circling in position and balance disturbance. The internal carotid artery of sham-operated rats was injected with 0.4 mL saline.

**Thrombolysis**
At 3 hours following blood clot injection, 0.4 mL saline and 1.5 × 10⁴ U urokinase were respectively injected into the caudal vein of rats in the model and urokinase groups.

**Neurological impairment following thrombolysis detected by NSS**
Ten rats each from the model and urokinase groups were tested 24 hours following thrombolysis. NSS[24] was used for assessment of neurological impairment. The NSS score ranges from 0 to 14. The higher the score, the more severe the injury. The mean value was obtained.

**Infarct volume as determined by TTC**
Ten rats were randomly obtained from each group at 24 hours following thrombolysis, anesthetized and sacrificed. At a low temperature, tissues, 2 mm anterior and posterior to the optic chiasma of the left brain[27-28], were serially sliced into 5 µm-thick coronal sections, placed in 2% TTC saline buffer and incubated at 37°C for 15 minutes. Infarct volume was calculated using MPIAS-500 Multimedia Color Pathologic Image Analysis System (Tongji Medical University, Wuhan, China).

**MMP-9 and TIMP-1 expression in the cerebral cortex, as detected by immunohistochemical staining**
Ten rats were randomly obtained from each group at 24 hours following thrombolysis. After anesthesia, the thoracic cavity was incised to expose the heart. Following left ventricular puncture, the right auricle was perfused with 250 mL heparinized saline, and then fixed in 250 mL 4% paraformaldehyde. The entire brain was placed in 4% paraformaldehyde for 48 hours, embedded in paraffin at 4°C, and then sliced into 6 µm-thick sections. The specimens were treated with xylene and ethanol, deparaffinized, and then incubated in 30 mL hydrogen peroxide for 5 minutes. After a wash in distilled water, sections were immersed in 0.01 M citrate buffer.
Antigens were retrieved by microwave heating. The sections were blocked with 5% bovine serum albumin for 20 minutes at room temperature, and then incubated with mouse anti-rat MMP-9 and TIMP-1 monoclonal antibodies (1:200; Boster, Wuhan, China) at 4°C overnight. Following three washes in 0.02 M PBS, each 3 minutes, the sections were incubated with biotinylated goat anti-mouse IgG (1:200; Boster) at 37°C for 30 minutes. Following three washes in PBS, each 3 minutes, the sections were incubated with streptavidin biotinylated peroxidase complex (Boster) for 30 minutes at 37°C. Following three washes in PBS, each 3 minutes, the sections were developed using diaminobenzidine, dehydrated in ethanol series, permeabilized in xylene, and mounted in neutral resin. PBS served as the negative control, in place of primary antibody. Absorbance values were quantitatively calculated using image pro plus software (Media Cybernetics, Bethesda, MD, USA).

MMP-9 and TIMP-1 mRNA expression in the cerebral cortex, as determined by in situ hybridization

Ten rats were randomly obtained from each group at 24 hours following thrombolysis. After anesthesia, the brain was dehydrated, embedded in paraffin, and sliced into 6–8-μm-thick sections. The slide was coated with polylysine. Sections were digested with pepsin diluted in 3% citric acid for 15 minutes at 37°C or room temperature. The sections were prehybridized, and then hybridized with digoxin-labeled MMP-9 and TIMP-1 oligonucleotide probes. The rat MMP-9 probe sequences are as follows: (1) 5'-TCC CTG CCC CAG ACT GGT GAG CTG TAT GAG ATG TGC GTC TTC CC-3'; (2) 5'-CAA CTC GGC AGA GAG CTG GAC AGC-3'; (3) 5'-CCA GGT GGA GAG CTG GAC AGC-3'; (2) 5'-CAA CTC GGC AGA GAG CTG GAC AGC-3'. The rat TIMP-1 probe sequences are as follows: (1) 5'-ACC ACC TTA TAC CAG CTG TAT GAG ATC AAG ATG AC-3'; (2) 5'-CAC AAG TCC CAG AAC CGC AGC GAG GAG TTT CTC AT-3'. The rat TIMP-1 probe sequences are as follows: (1) 5'-ACC ACC TTA TAC CAG CTG TAT GAG ATC AAG ATG AC-3'; (2) 5'-CAC AAG TCC CAG AAC CGC AGC GAG GAG TTT CTC AT-3'. The sections were incubated in hybridization solution at 38–42°C in a thermostat-regulated container. After the coverslip was removed, the sections were washed in 2 × sodium citrate buffer for 5 minutes at 37°C, twice, and then immersed in 0.5 × saline sodium citrate at 37°C for 15 minutes. The sections were incubated with blocking buffer at 37°C for 30 minutes, and then with biotinylated mouse anti-digoxin (1:500; Boster) at 37°C for 60 minutes, followed by four washes in PBS, each 5 minutes. The sections were incubated with streptavidin-biotin complex 37°C for 20 minutes, washed in PBS for 5 minutes, three times, incubated with biotinylated peroxidase (Boster) at 37°C for 20 minutes, and then washed in PBS for 5 minutes, four times. The sections were developed with diaminobenzidine, dehydrated in ethanol, permeabilized in xylene, and then mounted. Absorbance values were quantitatively calculated using image pro plus software.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA), and expressed as mean ± SD. Two-sample t-test was used. A value of $P < 0.05$ was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Medical College, Qingdao University, China.

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