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Brain distribution and elimination of recombinant human TIMP-1 after cerebral ischemia and reperfusion in rats

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Abstract

Objective—To investigate recombinant human TIMP-1 (¹²⁵I-rhTIMP-1) half-life in blood and its distribution in rat brain tissue after cerebral ischemia/reperfusion as part of a therapeutic development paradigm.

Method—A suture model of the middle cerebral artery occlusion was used. ¹²⁵I-labeled rhTIMP-1 at 60 µg/kg (11.23 µCi/µg) was administered to rats intravenously at the beginning of reperfusion. Blood and brain tissue were collected. The radioactivity was detected with a gamma counter and analyzed by autoradiography.

Results—The blood half-life $T_{1/2}$ of ¹²⁵I-rhTIMP-1 was 42.2 hours. Thirty minutes after ¹²⁵I-rhTIMP-1 administration, an increased accumulation of ¹²⁵I-rhTIMP-1 in the ischemic hemisphere was observed. The maximum brain tissue concentration C_{max} was 26.1 ng/g at 1.5 hours in the striatum and 13.9 ng/g at 5 hours in the cortex when the uptake percentage of brain tissue to blood was 6.1 ± 0.4 and $6.7 \pm 2.1\%$, respectively. The cortex and striatum elimination half-lives $T_{1/2}$ were 45.3 and 39.2 hours, respectively. Electrophoretic analysis of ischemic samples for ¹²⁵I-rhTIMP-1 showed a clear 28 kDa band 1.5 hours after ¹²⁵I-rhTIMP-1 administration in the cortex and striatum. The intensity of the 28 kDa band decreased after 3.0 hours of the administration. Some ¹²⁵I-rhTIMP-1 maintained its molecular integrity for 8.5 hours in ischemic striatum after reperfusion.

Discussion—¹²⁵I-labeled rhTIMP-1 was distributed quickly into ischemic brain tissue and had a slow elimination in both blood and brain tissue. These results, along with other studies suggesting therapeutic benefits, will aid in the development of TIMP-1 for protecting ischemic stroke.

Keywords

Tissue inhibitors of metalloproteinase-1; ¹²⁵I-radiolabeling; Autoradiography; Cerebral ischemia; Rat

Introduction

Strokes are a leading cause of death and disability worldwide. Accumulating data suggest that matrix metalloproteinase (MMP) activation and expression are important mediators in the pathophysiology of transient focal cerebral ischemia. More specifically, MMP-2 and MMP-9 have been shown to degrade components of the extracellular matrix, including

collagen and laminin in the basal lamina and zona occludens-1 of endothelial tight junctions, which results in blood brain barrier (BBB) breakdown, cellular edema, and neuronal death.¹ Previous studies have shown that ischemia/reperfusion exacerbates neurovascular injury by potentiating MMP-9 activation in the ischemic cortex and by activating MMP-2 in the ischemic striatum, which is related to hemorrhagic transformation (HT).^{2,3} Pharmacological inhibition of MMP-9 using MMP-9 gene knockout or anti-MMP-9 antibody significantly reduces proteolytic activity and tissue damage after cerebral ischemia in animal models.⁴ Blocking MMP-9 activation caused by ischemia/reperfusion is likely to be a feasible treatment to decrease ischemic brain injury.

MMP activity is tightly controlled by endogenous tissue inhibitors of MMPs (TIMPs), a family of glycosylated proteins comprised of four members (TIMP-1 to -4), which inhibit multiple MMPs and display different interactions and substrate specificity with MMP proenzymes. TIMP-1 is a 20.7 kDa glycoprotein, which is found either as a precursor, a free unbound protein, or in a complex with the active site of an MMP. It can also be found in a complex with pro-MMP-9, which prevents this pro-protein from becoming an active proteinase.⁵ MMP-9 protein expression and gelatinolytic activity were significantly increased 24 hours after transient focal cerebral ischemia in TIMP-1 knockout mice.⁶ Gene transfer of TIMP-1 and TIMP-2 using adenoviral vector-mediated delivery to mouse brains produced increased levels of TIMP-1 and TIMP-2 and reduced the extent of neuronal damage induced by transient global ischemia.⁷ In transgenic TIMP-1 mice with trauma or focal cerebral ischemia, MMP-9 levels were lower, brain lesion volume and infarction volumes were reduced, and BBB leakage was ameliorated by TIMP-1 over expression.⁸ These data provide proof that TIMP-1 has a neuro-protective role against acute brain injury, which may lead to its use as a therapeutic agent for stroke victims.

Endogenous TIMP-1 levels in normal adult rodent brains are very low. Although its expression may be induced early after focal and global ischemia, this may be inadequate to prevent increased MMP activity and brain cell death.⁹ The TIMP-1 pharmacokinetic data in animals were important to support the development of recombinant TIMP-1 (rTIMP-1) for the treatment of stroke victims. To date, few studies address rTIMP-1's pharmacokinetics in normal mice and rats.^{10,11} The present study was performed to elucidate the half-life of recombinant human TIMP-1 (rhTIMP-1) in blood and its distribution in brain tissue after transient focal cerebral ischemia in rats.

Materials and Methods

Preparation of iodinated rhTIMP-1

rhTIMP-1 was diluted in sterile, distilled water to a concentration of 0.4 mg/ml and stored at -80°C (Cat. no. PHC8023; Invitrogen Co., Carlsbad, CA, USA). rhTIMP-1 was iodinated with Na^{125}I (Cat. no. 016303702; MP Biomedicals, Inc., Irvine, CA, USA) using IODO-BEADS by following the manufacturer procedure (Cat. no. 28665; Thermo Fisher Scientific Inc., Rockford, IL, USA).¹² Two iodination beads were washed twice with 1 ml of 50 mM Tris buffer (pH 7.0) and dried on a Whatman filter paper. The beads were added to 125 μl of the same buffer containing 0.5 mCi of Na^{125}I (carrier free) for 5 minutes. Fifty micrograms of rhTIMP-1 was added, bringing the total volume to 250 μl . After 8.5 minutes of incubation at room temperature, the reaction was stopped by removing the solution from the reaction vial and transferring it to ZebaTM de-salt spin columns (Cat. no. 89889; Thermo Fisher Scientific Inc.) to remove unincorporated ^{125}I . The final average radioactivity of the material was 11.23 $\mu\text{Ci}/\mu\text{g}$, which was measured with a Packard Cobra II automatic gamma radiometer (Packard BioScience Co., Meriden, CT, USA).

Animal husbandry

Animal protocols were approved by the University of Cincinnati Animal Care Committee and conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Male Sprague-Dawley rats that weighed 290–320 g had unrestricted access to food and water and were housed in a controlled environment (19–26°C, 50±20% relative humidity, and a 12 hour light/dark cycle).

Rat focal cerebral ischemia model

Focal ischemia was produced using a previously optimized procedure,¹³ in which rats were anaesthetized with 3% isoflurane and maintained with 1.5% isoflurane in a mixture of 70% N₂ and 28.5% O₂. Rectal temperature was monitored and maintained at 37±0.5°C with a feedback controlled heating blanket. The left common carotid artery, external carotid artery, and internal carotid artery were isolated via a ventral midline incision. A 3/0 monofilament nylon suture was used to occlude the middle cerebral artery. The suture was inserted into the external carotid artery and advanced into the internal carotid artery until the tip occluded the junction of the middle cerebral artery and anterior cerebral artery. The wound was closed and the suture was kept in place for 3.5 hours. The rats were re-anesthetized, and the filament was pulled back to achieve reperfusion. After the operation, the rats were transferred to a temperature controlled incubator at 37°C for 20 minutes until they were completely awake, and then to cages with a Deltaphase Isothermal Pad (Braintree Scientific, Inc., Braintree, MA, USA) to prevent hypothermia. The rats were sacrificed at 4.0, 5.0, 6.5, 8.5, 12, 24, and 48 hours after the onset of ischemia (0.5, 1.5, 3.0, 5.0, 8.5, 20.5, and 44.5 hours after reperfusion).

Intravenous administration of ¹²⁵I-rhTIMP-1 to rats

Radiolabeled rhTIMP-1 in normal saline solution (59.35±3.75 µg/kg, 11.23 µCi/µg) was administered via a saphenous vein at the beginning of reperfusion. The experiments were repeated twice.

Collecting blood and brain tissue in rats

The rats were anesthetized with ketamine and xylazine followed by transcardial perfusion with 0.9% saline. At each sampling time point, two to four rats were used. Blood was collected via cardiac puncture except for the sample at 1 minute, which was withdrawn from the tail artery. Brain tissue (left and right hemispheres, cortex and striatum) was dissected and homogenized with lysis buffer. The radioactivity of blood and brain tissue was determined using a Packard Cobra II automatic gamma radiometer (Packard BioScience Co.). The specific radioactivity of ¹²⁵I-rhTIMP-1 in the ischemic hemisphere was calculated by subtracting the values from the contralateral hemispheres.

Analysis of ¹²⁵I-rhTIMP-1's brain tissue distribution

Brain tissues were weighed and then homogenized in the lysis buffer (5 mM ethylenediaminetetraacetic acid, 5 mM 1,10-phenanthroline, and 10 µl proteinase inhibitor cocktail) using a probe-type tissue homogenizer (Tekmar Tissumizer; Tekmar, Mason, OH, USA). The total radioactivity per gram of brain tissue was measured using a gamma counter. The tissue slurry was centrifuged at 300 g for 3 minutes at 4°C, then transferred to a 1.5 ml Eppendorf tube and centrifuged again at 12 000 g for 20 minutes at 4°C. The tissue homogenate supernatants and precipitations were stored at –20°C. The supernatants and precipitations were mixed with 2× protein loading buffer blue (EC-886; National Diagnostics, Atlanta, GA, USA), heated for 5.0 minutes at 98°C, and electrophoresed on

12.5% SDS-polyacrylamide gel for 1 hour at 150 V using the Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gel was visualized by autoradiography.

Data analysis

Pharmacokinetic parameters for the blood kinetics were determined from blood concentration–time profiles. Pharmacokinetic parameters for the cortex and striatum were estimated using the tissue concentration–time profiles. The apparent elimination rate constant k , the half-life $T_{1/2}$, and the area under the blood concentration–time curve were determined by non-compartmental analysis using WinNonlin 5.2.1 (Pharsight Corp., Mountain View, CA, USA). Half-lives were calculated as 0.693 divided by λ_z where λ_z is the terminal elimination rate constant. Clearance was calculated by dividing the injected dose by the area under the concentration–time curve, and the volume of distribution was calculated by dividing clearance by k . The maximum blood or tissue concentration $C_{\max}$ and the time of maximum blood or tissue concentration $T_{\max}$ were read directly from the plotted data. The radioactive ratio of brain tissue to blood for ^{125}I -labeled rhTIMP-1 was calculated as brain tissue radioactivity/blood radioactivity $\times 100\%$. All data are presented as the mean $\pm$ SD for the indicated number of animals.

Results

^{125}I -rhTIMP-1 blood pharmacokinetics

The concentration–time profile of ^{125}I -rhTIMP-1 in blood is shown in Fig. 1. Approximately 55% of the initial dose was distributed into the tissues 3 hours after intravenously administering ^{125}I -rhTIMP-1 (Fig. 1B). The blood half-life $T_{1/2}$ was 42.2 hours. The volume of distribution was 289.0 ± 18.3 ml/kg. The clearance was 4.7 ± 0.3 ml/hour/kg.

^{125}I -rhTIMP-1 brain distribution and elimination

The distribution and elimination of ^{125}I -rhTIMP-1 in ischemia/reperfusion cortex and striatum after its intravenous administration are illustrated in Fig. 2. An increased accumulation of ^{125}I -rhTIMP-1 in the ischemic hemisphere was observed 0.5 hours after administration. The maximum brain tissue concentration $C_{\max}$ was 26.1 ng/g striatum tissue at 1.5 hours and 13.9 ng/g cortex tissue at 5 hours after ^{125}I -rhTIMP-1 administration. The cortex and striatum elimination half-lives $T_{1/2}$ were 45.3 and 39.2 hours, respectively.

Uptake percentage of brain tissue to blood

The uptake percentage of brain tissue in ischemic hemisphere to blood is shown in Fig. 3. At the time points with the maximum brain tissue concentration $C_{\max}$ in cortex (5.0 hours) and striatum (1.5 hours), the uptake percentage of the brain tissue to blood was 6.7 ± 2.1 and $6.1 \pm 0.4\%$, respectively. The uptake percentage reached a maximum of 9.3% in the cortex and 10.5% in the striatum 20.5 hours after ^{125}I -rhTIMP-1 administration.

^{125}I -rhTIMP-1 integrity in brain tissues

Figure 4 shows the electrophoretic analysis of ischemic cortex and striatum samples for ^{125}I -rhTIMP-1. A clear 28 kDa band was observed 1.5 hours after ^{125}I -rhTIMP-1 administration in the cortex and striatum. The intensity of the 28 kDa band was decreased at 3.0 hours in the cortex and at 3.0, 5.0, and 8.5 hours in the striatum compared to its intensity 1.5 hours after administration when ^{125}I -rhTIMP-1 brain concentrations were still high (Fig. 2) and the uptake percentage of brain tissue to blood was gradually increased (Fig. 3).

Discussion

¹²⁵I-rhTIMP-1's blood half-life

In ischemic stroke, MMP expression and activation contribute to neurovascular unit injury and HT.^{14,15} TIMP-1 is an endogenous inhibitor of MMPs with the potential for reducing ischemic brain injury and HT occurrence.^{7,8} In the present study, we found that rhTIMP-1 had a long blood half-life (42.2 hours) when administered at the beginning of reperfusion [3.5 hours after middle cerebral artery occlusion (MCAO)]. The long blood half-life provides an extended time window for rhTIMP-1 to enter brain tissue after stroke.

A few studies have reported the pharmacokinetics of TIMPs in normal animals. Kurschat *et al.* injected ¹²⁵I-rTIMP-1 (555 kBq/μg, 0.15 μg/rat) intravenously into normal male Sprague-Dawley rats¹¹ and found that it disappeared from circulation within 2 minutes. Black's research team injected 17 ng of ¹²⁵I-labeled rhTIMP-1 into healthy BALB/c mice subcutaneously and collected blood samples 15 minutes to 24 hours after administration. The plasma concentrations of rhTIMP-1 climbed progressively for the first 60 minutes and peaked at 0.42 ng/ml (2.5% of initial dose/g). The distribution half-life was 58.6±14.0 minutes, and the blood elimination half-life was 4.11±0.35 hours.¹⁰ A possible explanation for these discrepancies is that a much lower dose of TIMP-1 was administered in the study conducted by Kurschat *et al.* and rapid distribution resulted in lower plasma drug concentration. Black *et al.* measured TIMP-1 plasma half-life in normal mice after intraperitoneal injection, while we measured TIMP-1's whole blood half-life in stroke-induced rats after intravenous injection. After cerebral ischemia, MMPs increased in blood and might bind blood cellular membranes. TIMP-1 binds the increased MMPs in blood and on these cell membrane with prolonged elimination half-life.¹⁶ The different routes of administration could also lead to different TIMP-1 distributions to blood and tissue.

¹²⁵I-rhTIMP-1 entering parenchyma

TIMP-1 is a 20.7 kDa glycoprotein (migrates as a 28.0 kDa protein on sodium dodecyl sulfate polyacrylamide gel electrophoresis gel due to glycosylation). TIMP-1 can prevent degradation of extracellular matrix and neuron death only if it passes through the BBB. Here, we report rhTIMP-1 distribution and elimination in ischemic brain tissue after intravenous injection of rhTIMP-1 at the beginning of reperfusion (3.5 hours after MCAO).

We found that radioactivity from ¹²⁵I-rhTIMP-1 appeared in brain tissue as early as 0.5 hours after administration. The radioactivity from ¹²⁵I-rhTIMP-1 increased and reached its maximum value 1.5 hours after reperfusion in the striatum and 5.0 hours after reperfusion in the cortex. The elimination of TIMP-1 was slow in both the cortex ($T_{1/2}$ =45.3 hours) and the striatum ($T_{1/2}$ =39.2 hours). The following evidence supports ¹²⁵I-TIMP-1 passing the BBB and entering the parenchyma during ischemic reperfusion: the radioactivity of ¹²⁵I-rhTIMP-1 reached a peak more quickly in the striatum than in the cortex; previous studies have shown that focal ischemia produced more severe injury in the striatum than in the cortex using the suture MCAO model.¹⁷ The uptake percentage of brain tissue to blood at the peak is 6.7% in the cortex 5 hours after reperfusion and 6.1% in the striatum 1.5 hours after reperfusion; however, the maximum uptake percentage of brain tissue to blood is 9.3% in the cortex and 10.5% in the striatum 20.5 hours after reperfusion. This suggests that TIMP-1 penetration into brain parenchyma increased when the ischemia/reperfusion period was prolonged with more severe BBB injury. A previous paper also showed that BBB permeability increased after ischemia/reperfusion and allowed passage of larger molecule weight substances into the brain. Albumin (66.43 kDa) passed the BBB 3.0 or 21.0 hours after reperfusion following 3.0 hours of ischemia.¹⁸ Therefore, rhTIMP-1 can pass the BBB and remain in brain parenchymal for a long time after cerebral ischemia/reperfusion. Since

the peak time of the HT and animal death was within 24–36 hours after cerebral ischemia/reperfusion,^{19,20} a single intravenous administration of rhTIMP-1 could maintain its brain concentration long enough to protect the brain from injury. However, only about 6–7% of TIMP-1 was taken into the brain from blood at the time points of maximum TIMP-1 concentrations in the cortex and the striatum ($T_{\max}$). The maximum uptake percentage was also low, only about 9–10% in the cortex and the striatum. Poor BBB penetration of TIMP-1 may limit its protection on basal lamina degradation and neuron death after ischemia/reperfusion.

¹²⁵I-rhTIMP-1 integrity in brain tissue

Although the TIMP-1 N-terminal domain is sufficient for inhibition of MMPs, the integral structure of TIMP-1 has the best effect on inhibiting MMP activity.²¹ Previous studies have shown that several kinds of proteinases were activated during ischemia/reperfusion and could proteolytically degrade and inactivate TIMP-1.^{22,23} Therefore, we assessed the molecular integrity of ¹²⁵I-rhTIMP-1 in ischemic brain tissue.

We found that bands of ¹²⁵I-rhTIMP-1 corresponding to approximately 28.0 kDa were visible at 1.5, and 3.0 hours in the cortex and 1.5, 3.0, 5.0, and 8.0 hours in the striatum after reperfusion. These data suggest that some TIMP-1 maintains its integral structure in brain tissue for at least 8.5 hours after reperfusion. However, the intensity of the 28 kDa bands was weaker 3 hours after reperfusion when ¹²⁵I-rhTIMP-1 brain concentrations were still high and uptake percentage of brain tissue to blood was gradually increased. This suggests that the degradation of TIMP-1 occurred in ischemia/reperfusion brain tissue. Although we performed autoradiography on supernatant and precipitation of brain tissue, bands were observed only from precipitation, which further confirmed that most of the ¹²⁵I-rhTIMP-1 were bound to the matrix and to cell surfaces.²⁴

In summary, we have reported the pharmacokinetics of ¹²⁵I-rhTIMP-1 in blood and brain tissue by administering ¹²⁵I-rhTIMP-1 intravenously after cerebral ischemia and reperfusion. ¹²⁵I-rhTIMP-1 was distributed quickly to ischemic brain tissue and had a slow elimination in both blood and brain tissue. Some ¹²⁵I-rhTIMP-1 maintained its molecular integrity for 8.5 hours in ischemic striatum after reperfusion. These results, along with other studies suggesting therapeutic benefits, will aid in the development of TIMP-1 as a drug for protecting ischemic stroke victims. However, the poor BBB penetration and the degradation of ¹²⁵I-rhTIMP-1 in ischemic brain tissue may restrict its usage in future stroke treatment.

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Figure 1.

Time-concentration curve of ^{125}I -rhTIMP-1 in blood after focal cerebral ischemia and reperfusion in rats. (A) ^{125}I -rhTIMP-1 blood concentration changes over time. (B) The percentage of blood ^{125}I -rhTIMP-1 distribution and elimination over time.

Figure 2.
Time-concentration curve of ^{125}I -rhTIMP-1 in (A) the cortex and (B) striatum after focal cerebral ischemia and reperfusion in rats.

Figure 3. The uptake percentage of brain to blood for ^{125}I -rhTIMP-1 in the (A) cortex and (B) striatum after focal cerebral ischemia and reperfusion in rats.

Figure 4.
The electrophoretic analysis of ^{125}I -rhTIMP-1 in ischemic (A) cortex and (B) striatum after focal cerebral ischemia and reperfusion in rats.