Edaravone, a Free Radical Scavenger, Inhibits MMP-9–Related Brain Hemorrhage in Rats Treated With Tissue Plasminogen Activator

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Background and Purpose—Intracerebral hemorrhage, induced by recombinant tissue plasminogen activator (rtPA) in ischemic stroke, is attributable to the increased activity of matrix metalloproteinase-9 (MMP-9). Patients with acute infarct benefit from the neuroprotective drug edaravone, a free radical scavenger. We examined the mechanisms by which edaravone may help to suppress rtPA-induced brain hemorrhage.

Methods—Male Wistar rats weighing 250 to 280 g were subjected to 3-hour transient middle cerebral artery occlusion (MCAO) and divided randomly into 3 groups. Immediately after reperfusion, 1 group was intravenously injected with 10 mg/kg rtPA, another with rtPA plus 3 mg/kg edaravone, and the 3rd group received no treatment. We assessed the hemorrhage volume and the activity of MMP-9 in the brain 24 hours postischemia. We also studied the activity of MMP-9, its mRNA expression, and nuclear factor-kappa B (NF-κB) activity in rtPA-stimulated human microvascular endothelial cells (HBECs).

Results—The degree of hemorrhage and the level of endothelial cell–derived MMP-9 were elevated in rats treated with rtPA alone and attenuated in rats treated with rtPA plus edaravone. In rtPA-stimulated HBECs, edaravone suppressed the activity and mRNA expression of MMP-9 in a dose-dependent manner. Edaravone also inhibited NF-κB activation.

Conclusions—We demonstrate that edaravone inhibits rtPA-induced cerebral hemorrhage in the ischemic brain of rats via the inhibition of MMP-9 expression in vivo, which is substantiated by inhibition of MMP-9 expression and NF-κB activation in HBECs. Edaravone may render thrombolytic therapy safer for the administration of rtPA in patients with ischemic stroke. (Stroke. 2009;40:626-631.)

Key Words: MMP-9 ■ brain hemorrhage ■ tissue plasminogen activator ■ free radical scavenger ■ cerebral infarction

The intravenous (iv) administration of recombinant tissue plasminogen activator (rtPA) within 3 hour of symptom onset is recommended in patients with ischemic stroke.1,2 Although its fibrinolytic activity is beneficial, rtPA must be delivered within a narrow time-window and it can induce several types of brain injury; it is neurotoxic, may lead to hemorrhagic transformation, and may exacerbate brain edema.3,4 For the optimal application of rtPA these issues must be addressed. MRI- rather than standard computed tomography–based thrombolysis may be one of the keys for solving these problems because it may help to decrease the morbidity rate of patients with symptomatic brain hemorrhage who were treated longer than 3 hour postonset.5 Another key may consist of combination therapy comprising rtPA plus agents that block its negative effects.3

The rtPA-induced increase in MMP-9 activity in the ischemic brain raises the risk for neurovascular cell death, brain edema, and hemorrhage due to disruption of the blood brain barrier (BBB).3,6 The inhibition of MMP-9 may make the rtPA-treatment of ischemic stroke safer.3,7

In Japan, edaravone, a free radical scavenger, is used in patients with acute ischemic stroke.1,8 In a randomized controlled trial, patients with acute ischemic stroke who were treated with edaravone had improved functional outcomes.8 Free radicals produced in the ischemic brain may result in membrane injury of neurons and endothelial cells.9 Edaravone may prevent such cell injuries and suppress neuronal death and brain edema by scavenging free radicals such as a peroxy- and hydroxyl radicals and by inhibiting lipid peroxidation.10–12 We previously demonstrated oxidant damage in ischemic rat brain lesions13; in patients with acute cerebral infarction, oxidative brain damage was inhibited by edaravone.14

In a rat model, edaravone inhibited rtPA-induced intracerebral hemorrhage.15 Another free radical scavenger, NXY-059, also reduced rtPA-induced brain hemorrhage in humans;
however, it is not approved for use in patients with ischemic stroke because it failed to significantly improve the neurological outcome in a randomized trial. To date, the mechanism(s) underlying the beneficial effects of edaravone against rtPA-induced brain hemorrhage remains unknown. Therefore, we examined the potential of edaravone to attenuate rtPA-induced hemorrhage in the ischemic rat brain and studied its functional mechanism(s) by focusing on its regulation of MMP-9 activity and suppression of nuclear factor kappa B (NF-κB) activation in brain endothelial cells.

**Materials and Methods**

**Transient Cerebral Ischemia Model**

In all experiments we used a protocol approved by the Animal Care Committee of Tokushima University Medical School. All surgical procedures were with the rats under anesthesia with 2% isoflurane in 30% oxygen and 70% nitrous oxide. During the procedures, their rectal temperature was monitored with a thermometer (KN-91, Natsume) and maintained at 37±0.5°C with a warming plate. We performed 3-hour middle cerebral artery occlusion (MCAO) by inserting an intraluminal filament as described previously. Briefly, male Wistar rats weighing 250 to 280 g were anesthetized and the right occipital artery and the external carotid artery (ECA) were ligated and isolated. To block major collateral flow the pterygopalatine artery at a site distal to the ligation was ligated and isolated. This procedure resulted not only in a severe brain ischemia but also in blood clot formation in the pterygopalatine artery at a site distal to the ligation.

The internal- and the common carotid arteries were transiently occluded with loosely tied 3-0 silk sutures; a silicon-coated 4-0 nylon thread was introduced into the ECA and advanced into the internal carotid artery to occlude the proximal orifice of the middle cerebral artery (MCA). To confirm MCAO, blood flow in the MCA was measured from the temporal bone surface at a site 1 mm posterior to the bregma and 3 mm inferior to the temporal line using a laser-Doppler flow probe (Unique Medical). After MCAO, it was reduced to 20% to 30% of the baseline. At the site of probe placement the temporal bone was thin enough to allow measuring the cerebral blood flow without any bone treatment. Performing this measurement at the nontreated skull surface was less invasive, and we preliminarily confirmed that this was as effective a way to monitor the degree of induced cerebral ischemia as measurement through a hole or a thinned skull area produced with a drill. Rats with successful MCAO consistently exhibited circling behavior, decreased resistance to lateral push, forelimb flexion, and shoulder adduction. They were reanesthetized and reperfused by slowly pulling the thread back after 3-hour MCAO. Before reperfusion, rats with incomplete MCAO (approximately 10%) were excluded from further study by a blinded observer. Blood pressure was measured by telemetry (Data Science) before, during, and after MCAO.

**rtPA- and Edaravone Administration**

After MCAO, the rats were randomly distributed among 3 groups of 16 rats each; group 1 was nontreated, group 2 was treated with rtPA alone, and group 3 received both rtPA and edaravone. rtPA (Alteplase, the active ingredient of Actilyse, a glycoprotein) and edaravone were a gift from Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan).

Immediately after reperfusion group 2 underwent the 20-minute iv administration of rtPA solution (10 mg/kg body weight in 0.75 mL saline) with an infusion pump. Group 3 was given an edaravone bolus (3 mg/kg, 3 mg/mL) followed by the iv injection of rtPA. Group 1 served as the MCAO control and was injected with saline. Whereas rtPA alone produces BBB disruption, the activation of rtPA or the formation of thrombolytic products after its binding to thrombin plays an important role in the disruption of the BBB. As thrombus formation is required in animal models, we activated rtPA with an autologous blood clot according to a previously established procedure. Briefly, a blood clot formed in an 18-mm PE-50 catheter was rinsed in saline to remove red blood cells and then incubated with rtPA solution. We posited that the administered rtPA is activated again by the presence of the autologous thrombus in the pterygopalatine artery and able to contact the blood clot in the artery from the opened distal side.

**Tissue Processing**

At 24 hours after the induction of focal cerebral ischemia the rats were euthanized and transversely perfused with cold saline. Their brains were removed immediately and brain tissue from an area 4 mm anterior and 8 mm posterior to the bregma was cut into 6 serial 2-mm coronal sections. In our preliminary study we found that among all slices, the reproducibly largest infarct size was in the cortical region of the right hemisphere on the anterior 3rd slice. Therefore, we used this area to analyze the extent of intracerebral hemorrhage and MPP-9 activity. The samples were stored at −80°C until use.

**Measurement of the Infarct Volume**

Sliced brain tissues were immersed in a 2,3,5-triphenyltetrazolium chloride solution in phosphate-buffered saline (PBS). The extent of ischemic infarction was traced and the integrated volume was calculated using NIH J 1.36b image software. The artifact from brain edema was reduced by the indirect measurement method based on the contralateral brain volume.

**Spectrophotometric Assay for Intracerebral Hemorrhage**

The hemoglobin content of extravasated blood in brain tissue affected by our experimental procedures was quantified by spectrophotometric assay. After adding 250 μL of water to individual samples they were homogenized for 30 seconds (ISO Inc), sonicated for 1 minute on ice with a pulse ultrasonicator (Tomiy Seiko Co), and centrifuged at 13 000 rpm for 30 minutes (Tomiy Seiko). Using bovine serum albumin as the standard, the protein content in each supernatant was determined with the BCA kit (Pierce) and adjusted to be the same (10 μg) in each sample. The hemoglobin-containing supernatant was collected, 80 μL of Drabkin reagent (Sigma Diagnostics; K$_3$Fe[CN]$_6$, 200 mg/L; KCN, 50 mg/L; NaHCO$_3$, 1 g/L; pH 8.6) were added to 20-μL supernatant aliquots and the sample was allowed to stand for 15 minutes at room temperature. The optical density (OD) in each group (n=8) was measured at 540 nm and expressed as the amount of cyanothemoglobin converted from hemoglobin. As the procedures yielded a linear relationship between the OD and the volume of blood added to the perfused normal brain of a sham-operated rat, it was used as the standard curve. Data are presented in terms of the hemorrhage volume (μL).

**Assay of MMP-9 Activity**

To extract protein from the tissue, it was minced and homogenized on ice in lysis buffer (50 mmol/L Tris-HCl, pH 7.6; 1.5 mmol/L NaCl, 0.5 mmol/L CaCl$_2$, 1 μmol/L ZnCl$_2$, 0.01% Brij 35, and 1.0% Triton X-100) and centrifuged at 4°C for 20 minutes at 9000 rpm. Supernatants were divided into aliquots and stored at −80°C. To measure MMP-9 activity, each sample was adjusted to the same protein content. We followed the instructions of the manufacturer of the MMP-9 Biotrak activity assay system kits (Amersham Biosciences UK Limited) to assess MMP-9 activity in brain extracts. All data for each group (n=8) are shown as the increase in MMP-9 activity after subtracting the MMP-9 activity in the normal brain of a sham-operated rat.

**Immunohistochemistry**

Brain tissue was immersed overnight in 4% paraformaldehyde in PBS and cut into 6-μm-thick frozen sections. After blocking with serum-free protein block (Dako Cytomation) for 15 minutes, primary antibodies diluted with Canget signal immunostain (Toyobo) were added for 1-hour incubation at room temperature. The antibodies were rabbit polyclonal antirat MMP-9 (1:200 dilution, Chemicon) and goat polyclonal anti-mouse platelet endothelial adhesion mole-
Gelatinase Zymography

After 12-hour rtPA treatment, the conditioned culture medium was centrifuged (10 000g, 2 minutes), and the supernatant was concentrated on a Microcon centrifugal filter device (Millipore). The samples were assayed for gelatinase activity by zymography, subjected to electrophoresis in polyacrylamide gels containing 0.5 mg/mL gelatin in the presence of SDS under nonreducing conditions, and incubated at 37°C for 24 hours in 100 mmol/L Tris-HCl (pH7.4) and 10 mmol/L CaCl2. Then they were stained with Coomassie Brilliant Blue R-250 and destained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activity appeared as clear bands against a blue background; they were measured using a computer image analysis system (NIH Image J).

RT-PCR

After 4-hour treatment of HBECs with rtPA, total RNA was isolated with the BioRobot EZ1 and EZ1 Universal tissue kit (Qiagen), and simultaneously treated with DNaseI (Qiagen). RNA was converted into cDNA using the transcriptor first-strand cDNA synthesis kit (Roche Diagnostics). The RT-PCR conditions were: 95°C for 10 minutes followed by 35 cycles at 96°C for 1 minute, 64°C for 1 minute, and 68°C for 1 minute. The final step was 68°C for 7 minutes. The cycling condition for GAPDH was similar; the annealing step was performed at 65°C for 1 minute. The primers were 5'-CCGAGGCG-CCTTTCCTAT-3' for human GAPDH and 5'-TGAACGGAAGCTCAGGG-3' for human MMP-9. The amplified product was separated on 1.5% agarose gels containing EtBr solution (Wako) and visualized on an ultraviolet transilluminator. The results were quantified after normalization to the expression of GAPDH mRNA.

Western Blot Analysis

After 4-hour rtPA treatment, HBECs were harvested, immediately sonicated in RIPA buffer (Pierce, Rockford, USA) containing phosphatase- and protease- inhibitors (Roche, Tokyo, Japan), and centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatants were stored at −80°C until use. The protein content in each sample was adjusted to be identical. Protein was separated by 7.5% or 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blocking was with 5% skim milk or BSA in Tris-buffered saline solution-Tween 20 (T-TBS). The samples were then incubated with the primary antibodies in Canget signal immunoblotting solution (IX71SIF-2, Olympus).

Cell Culture

We used human brain microvascular endothelial cells (HBECs; Cell Systems, Kirkland, Wash) from passages between 4 and 6; they were serially seeded on 60-mm collagen type-I coated dishes (Iwaki) using CS-C complete medium (Cell Systems). At subconfluence the medium was exchanged for serum-free medium containing growth factor but no other hormones and phenol red (Cell Systems) and incubation was continued for 2 days. Edaravone (0 to 100 μmol/L) was added to the HBECs 30 minutes before the addition of rtPA (20 μg/mL). The optimal length of rtPA exposure was determined with time-course experiments (data not shown). Data are the result of 3 independent-experiments.

Data Analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey-Kramer test. Data were expressed as the mean±SD. Differences of P<0.05 were considered statistically significant.

Results

Blood Pressure

The 3 groups did not differ significantly with respect to their blood pressure before- (MCAO control versus rtPA versus rtPA+edaravone; mean±SD: 109.8±3.5 versus 106.6±2.1 versus 109.5±3.4 mm Hg), during- (127.9±9.2 versus 128.1±6.3 versus 128.8±3.2 mm Hg), and after MCAO (115.8±2.1 versus 118.6±7.8 versus 114.8±4.5 mm Hg).

Infarct Volume

In our 3-hour MCAO model, the infarct volume in neither the whole brain nor the cortical region on the anterior 3rd slice was significantly different among the 3 experimental groups (MCAO control: 286±81, rtPA: 264±79, rtPA+edaravone: 268±84 mm³, MCAO control: 50.3±6.8, rtPA: 43.0±11.3, rtPA+edaravone: 44.1±3.2, respectively). Therefore we considered the results of analysis of this section to be independent of the extent of infarction.
Assay of Intracerebral Hemorrhage
The hemorrhage volume in the transiently ischemic brain was evaluated by the hemoglobin content of extravasated blood (Figure 1-A). The degree of hemorrhage in MCAO rats treated with rtPA was significantly higher than in the MCAO controls \(P<0.01\); it was significantly lower in rats injected with both rtPA and edaravone \(P<0.01\). Our results demonstrate that edaravone effectively inhibited rtPA-induced cerebral hemorrhage in the ischemic rat brain.

MMP-9 Activity
Next we examined the effect of edaravone on MMP-9 activity (Figure 1B). In the ischemic brain, the expression of MMP-9 was remarkably upregulated by rtPA \(P<0.01\). On the other hand, when MCAO rats were treated with rtPA plus edaravone, MMP-9 activity was significantly decreased \(P<0.01\). The activity of MMP-9 paralleled the degree of brain hemorrhage. These findings suggest that edaravone suppressed rtPA-induced intracerebral hemorrhage by reducing the activity of MMP-9.

Immunohistochemistry
To identify cells that express MMP-9 after rtPA treatment we assessed ischemic and nonischemic brain regions immunohistochemically. rtPA treatment increased MMP-9–positive cells in the ischemic lesion (Figure 2); no remarkable increase in MMP-9–positive cells was recognized in the nonischemic part (data not shown). MMP-9–positive cells were colocalized with PECAM-1–positive endothelial cells. These findings show that rtPA-induced MMP-9 was primarily derived from injured endothelial cells.

Regulation of MMP-9 Activity in HBECs
We studied the mechanisms underlying the regulation of MMP-9 by rtPA and edaravone in HBECs. Zymographic analysis of culture medium showed that HBECs stimulated with rtPA exhibited significantly increased MMP-9 activity, and additional treatment with edaravone suppressed this activity in a dose-dependent manner (Figure 3A). Semiquantitative RT-PCR disclosed that the mRNA level of MMP-9 was higher in rtPA-treated than rtPA-untreated HBECs (Figure 3B). The rtPA-induced increase in MMP-9 mRNA was inhibited by simultaneous exposure to edaravone. These findings suggest that edaravone suppressed rtPA-induced MMP-9 activity partly via the regulation of transcriptional synthesis.

We also examined the effect of edaravone on NF-κB, a main transactivator for MMP-9 expression in rtPA-treated HBECs. (Figure 4-A and 4B) The rtPA-induced increase in the activated form of NF-κB was reduced by the addition of edaravone. On the other hand, total NF-κB or IκBα expression was not significantly affected by rtPA with or without edaravone. In HBECs, edaravone may control the rtPA-
induced increase in MMP-9 activity by inhibiting the activation of NF-κB.

Discussion

Our study demonstrates that edaravone suppresses rtPA-induced MMP-9 upregulation and hemorrhage in the transiently ischemic rat brain and that in HBECs it inhibits rtPA-induced NF-κB activation and the elevation of MMP-9 activity.

MMP-9 mediates tPA-related BBB disruption after brain ischemia.3,20 In our and previous studies, rtPA-induced MMP-9 was identified primarily in endothelial cells in the ischemic brain.3,20 Based on these findings we posit that edaravone prevents rtPA-related hemorrhage in the ischemic rat brain by the inhibition of MMP-9–induced BBB disruption.

As the infarct size was not different among our groups of rats treated with rtPA or edaravone, the effect of rtPA and edaravone on the degree of hemorrhage in the ischemic brain could be evaluated independent of the infarct volume. However, in patients with acute ischemic stroke, rtPA is used to achieve the early recanalization of an occluded artery, resulting in a reduction in the size of the cerebral infarct.1,2 Edaravone also inhibits neuronal death and the extent of cerebral infarction in rats.10,11,15,21 There is a discrepancy between our and previous studies. We posit that the difference in the effect of rtPA is attributable to mechanical recanalization after MCAO irrespective of the delivery of thrombolytic therapy. The pterygopalatine artery, a well-developed anastomotic branch to the cerebral artery in rodents, was occluded in our study but not in other studies. Therefore, the volume of the reversible ischemic brain injury rescued by edaravone may be smaller in our model than in other models. Moreover, we administered edaravone immediately after reperfusion; in other studies it was delivered earlier or more frequently.10,15,21

The effect of edaravone on hematoma-related delayed neuronal death was not accessed in our study, but edaravone may inhibit it by suppressing not only rtPA-induced hemorrhage but also iron- and thrombin-induced cell injuries directly.22

Figure 4. Western blot analysis of NF-κB and AP-1 pathway activation in HBECs exposed for 4 hours to rtPA (20 μg/ml) in the presence or absence of edaravone (A). The expression of the p65 subunit in activated NF-κB (active p65) is increased by rtPA (P<0.01) and decreased by the combination of rtPA and edaravone (P<0.01). The expression of total NF-κB (total p65) and IκBα is not significantly affected by rtPA or edaravone. The data are the result of 3 measurements in 3 independent experiments and are shown as the relative increase over nontreated HBECs (mean±SD). **P<0.01. VC indicates vehicle control.

Occluded brain arteries are reperfused via rtPA-induced thrombolysis.1 There has been a focus on the pathway of rtPA-induced but not on endogenous tPA-mediated hemorrhage.3,7,15 Spontaneous brain hemorrhages in brain infarctions are rare compared to hemorrhages after rtPA treatment, and the underlying mechanism(s) may be different between spontaneous hemorrhages and bleeding induced by rtPA. Hosomi et al23 reported that in their rat model of transient brain ischemia, endogenous tPA activity was not upregulated because the level of plasminogen activator inhibitor (PAI) was increased, resulting in the PAI–PAI-1 complex. In rabbits with brain ischemia induced with a blood clot, the nonspecific MMP-9 inhibitor BB-94 suppressed hemorrhage induced with rtPA, however it had no significant effect on spontaneous hemorrhage.7 The current study demonstrated only the ability of edaravone to inhibit rtPA-induced hemorrhage. Further investigations are required to determine whether it might be able to suppress spontaneous hemorrhage unrelated to rtPA.

Previous studies demonstrated that in HBECs the rtPA-induced upregulation of MMP-9 was mediated by the low-density lipoprotein receptor–related protein (LRP) but not the NMDA receptor,20 even though rtPA enhances NMDA-mediated neuronal death.4 Furthermore, it was reported that rtPA mediated MMP-9 expression through NF-κB activation, which could promote MMP-9 gene transcription via binding to its promoter,20 and our in vitro findings are consistent with these observations.

Edaravone, an antioxidant and free radical scavenger, exerts antiinflammatory effects by inhibiting inflammatory cytokines including IL-1β, IL-6, and TNF-α in various diseases.24,25 In IL-1β–stimulated synovial cells, its antiinflammatory activities prevent the nuclear translocation of NF-κB.26 Another free radical scavenger, α-lipoic acid, inhibits MMP-9 expression by inhibiting NF-κB transcriptional activity in TNF-α–stimulated vascular smooth muscle cells.27 These earlier findings support our hypothesis that edaravone inhibits NF-κB activation via its antioxidant properties. As the results of our in vitro study cannot be completely extrapolated to in vivo conditions, we cannot rule out other mechanisms that may contribute to the effects of edaravone on the rtPA-treated ischemic brain.

Cheng et al3 showed that activated protein C prevented rtPA-induced hemorrhage in ischemic rat brains. They reported that like edaravone, it blocked the rtPA–NF-κB–MMP-9–hem-
orrhage pathway. At present, activated protein C is not available to treat patients with ischemic stroke. On the other hand, the clinical safety of edaravone has been established. Clinical trials are needed to confirm that edaravone can prevent rtPA-induced cerebral hemorrhage. In combination, rtPA and edaravone therapy may lead to the early recanalization of occluded cerebral arteries while suppressing brain hemorrhage, and this may constitute safer thrombolytic therapy.

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rtPA (Alteplase) and edaravone were a gift from Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan).

Disclosures

None.

References

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