Rapid Breakdown of Microvascular Barriers and Subsequent Hemorrhagic Transformation After Delayed Recombinant Tissue Plasminogen Activator Treatment in a Rat Embolic Stroke Model

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Background and Purpose—Thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) after stroke increases risk of hemorrhagic transformation, particularly in areas with blood-brain barrier leakage. Our aim was to characterize acute effects of rtPA administration on the integrity of microvascular barriers.

Methods—Stroke was induced in spontaneously hypertensive rats by unilateral embolic middle cerebral artery occlusion. Six hours after stroke, rtPA was intravenously administered (n = 10). Controls received saline (n = 4). Extravasation of the large-diameter contrast agent monocrylalline iron oxide nanocolloid (MION) was assessed with susceptibility contrast-enhanced MRI during rtPA injection. In addition, we performed perfusion MRI and diffusion-weighted MRI. After MRI, 2 hours after rtPA treatment, intracerebral hemorrhage was quantified with a spectrophotometric hemoglobin assay.

Results—Late rtPA treatment resulted in increased hemorrhage volume (8.4 ± 1.7 versus 2.9 ± 0.9 μL in controls; P < 0.05). In MION-injected animals, during rtPA administration, transverse relaxation rate change (ΔR₂*) increased from 12.4 ± 6.0 to 31.6 ± 19.2 s⁻¹ (P < 0.05) in areas with subsequent hemorrhage. Significant ΔR₂* changes were absent in nonhemorrhagic areas, in animals without injected MION, and in saline-treated animals. Thrombolytic therapy did not improve perfusion in regions with hemorrhagic transformation (cerebral blood flow index was 22.8 ± 19.7% [of contralateral] at 0.5 hours before and 22.4 ± 18.0% at 1 hour after rtPA administration).

Conclusions—The ΔR₂* changes during rtPA delivery in MION-injected animals indicate extravasation of MION, which reflects increased permeability of the blood-brain barrier. This implies that late rtPA treatment rapidly aggravates early ischemia-induced damage to microvascular barriers, thereby enhancing hemorrhagic transformation. (Stroke. 2002;33: 2100-2104.)

Key Words: blood-brain barrier ■ cerebral hemorrhage ■ cerebral ischemia, focal ■ magnetic resonance imaging ■ thrombolytic therapy ■ rats

Thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) after acute ischemic stroke has been shown to be effective when applied within 3 hours after onset of symptoms. Conversely, rtPA administration leads to increased risk of hemorrhagic transformation. Understanding the risks and basis of bleeding may improve the efficacy of treatment with rtPA. We have previously demonstrated in a rat embolic stroke model that rtPA-enhanced hemorrhagic transformation emerges in areas that exhibit early blood-brain barrier (BBB) perturbation before rtPA treatment. With the use of postcontrast T1-weighted MRI, enhanced leakage of the contrast agent Gd-DTPA before rtPA administration was found in areas with subsequent hemorrhage after rtPA treatment. Our previous study demonstrated the potential of MRI to provide information on risks of hemorrhagic transformation before onset of rtPA therapy. However, the process through which rtPA enhances hemorrhagic transformation after onset of therapy remains unclear. In the present study the main goal was to characterize the acute, direct effects of rtPA administration on the integrity of the microvasculature. To that aim we assessed extravasation of an intravascular contrast agent with a larger diameter than Gd-DTPA by use of steady state susceptibility contrast-enhanced MRI during rtPA injection after embolic stroke in rats. In addition, perfusion MRI and diffusion-weighted MRI were performed to characterize hemodynamics and tissue changes, respec-
ty, and intracerebral hemorrhage was evaluated anatomically and quantified spectrophotometrically.

Materials and Methods

Animal Model

Experimental protocols were institutionally approved in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Unilateral stroke was induced in male spontaneously hypertensive rats (weight, 300 to 350 g) (Taconic, Germantown, NY) by embolic occlusion of the right middle cerebral artery (MCA), as previously described. Animals were anesthetized with halothane (1% to 1.5%) in \( O_2/N_2O \) (1/2) under spontaneous respiration. Body temperature was maintained at 37°C to 38°C. A modified PE50 catheter with a 50-mm-long homologous blood clot was inserted into the right external carotid artery and advanced into the internal carotid artery until the tip was positioned just proximal to the origin of the MCA. The next clot was carefully injected into the internal carotid artery. Once injected intravascularly, the string-shaped clot will be compactly agglutinated. After 5 minutes, the catheter was withdrawn from the external carotid artery.

Before MRI, rats were tracheotomized and mechanically ventilated with 1% halothane in \( O_2/N_2O \) (1/1). The right femoral artery was catheterized for monitoring of arterial blood pressure, \( P_{CO_2}, P_{O_2} \), and pH. The right femoral vein and jugular vein were cannulated for administration of therapeutic agent and MR contrast agent, respectively.

Six hours after MCA occlusion, human rtPA (Genentech Inc) was intravenously administered (a dose of 10 mg/kg of a solution containing 2 mg/mL; 10% bolus and the remainder continuously infused over 20 minutes). Control animals received saline. The relatively high dose of rtPA used here is based on the 10-fold difference in enzyme activity in rodent versus human systems. Despite the high dose, we did not observe profuse bleeding from the surgical site, although this may be due in part to minimal surgery involved in this model.

Magnetic Resonance Imaging

MRI was done on a 2.0-T magnet system (SISCO/Varian Instruments) with the use of a surface coil (30-mm diameter). During the MRI experiments body temperature, blood pressure, and blood gases were carefully controlled and maintained at normal values.

Multislice diffusion-weighted MRI (repetition time [TR]/echo time [TE] = 2000/40 ms; b = 150, 850, 1550 s/mm\(^2\); diffusion-encoding gradients in 3 directions) was performed (field-of-view [FOV] = 25\( \times \)25 mm\( ^2 \); 64\( \times \)64 data matrix; nine 1.5-mm slices). Two-dimensional maps of the mean trace of the apparent diffusion coefficient (ADC) of tissue water were calculated from the diffusion-weighted MRI data.

Second, single-slice dynamic susceptibility contrast-enhanced MRI (gradient echo echo-planar imaging; TR/TE = 175/22 ms; FOV = 25\( \times \)25 mm\( ^2 \); 32\( \times \)32 data matrix, zero-filled to 64\( \times \)64; slice thickness 1.5 mm; 500 consecutive images) was done in combination with intravenous bolus injections of monocrystalline iron oxide nanocolloid (MION) (up to 5 mg/kg), from which we calculated the signal intensities at time \( t \) and before injection of contrast agent, respectively.

Spectrophotometry

After the MRI experiments (approximately 8 hours after ischemia and 2 hours after rtPA), rats were killed by a lethal overdose of sodium pentobarbital and transcardially perfused with phosphate-buffered saline. Brains were removed and cut in 2-mm coronal sections. The anatomic distribution of intracerebral hemorrhage was estimated on unstained brain sections, and the hemorrhage volume was quantified with a spectrophotometric hemoglobin assay. With this method, hemorrhage volume is calculated from the linear relationship between optical density of homogenized hemispheric brain tissue and hemoglobin concentration. Additionally, to test the effect of intracerebral MION accumulation on this correlation, we calculated standard absorbance curves after adding MION (0 mg/mL, 0.05 mg/mL, and 0.5 mg/mL, and 0.5 mg/mL in 10 mL), along with incremental volumes of homologous blood (0, 5, and 10 mL), to homogenized hemispheric brain tissue of control rats. A total of 9 samples were analyzed, each with a different MION concentration and blood volume.

Experimental Protocol

Diffusion-weighted and perfusion MRI were done between 1 hour before and 2 hours after rtPA (n = 10) or saline (n = 4). Steady state susceptibility contrast-enhanced MRI was performed during administration of rtPA or saline. Animals that were treated with rtPA were divided into 2 experimental groups. One group of animals was given MION injection during the MRI protocol (n = 6), and a second group of rtPA-treated animals did not receive MION (n = 4). All saline-treated animals received MION. Immediately after MRI, brains were removed for morphological analysis and spectrophotometric assay of intracerebral hemorrhage.

Data Analysis

Regions of interest (ROIs) on MR images (9 voxels; 2.1 mm\(^3\)) were outlined in regions that spatially correlated with areas showing clear accumulation of extravascular blood on the unstained brain sections. In addition, ROIs were outlined in regions within the lesion area with no hemorrhagic transformation. Finally, anatomically corresponding ROIs in the contralateral hemisphere were selected.

Statistical comparisons were performed with the use of ANOVA with post hoc Bonferroni t-test. Probability values of <0.05 were considered significant.

Results

Embolic occlusion of the MCA resulted in an ischemic lesion that was characterized by perfusion loss and reduced tissue water ADC in the ipsilateral MCA territory (cortex and striatum). Treatment with rtPA at 6 hours after ischemia clearly enhanced hemorrhage. Excised brain sections after the MRI experiments demonstrated intracerebral hemorrhage in both cortical and subcortical regions in the ischemic territory of all animals that received rtPA. Signs of hemorrhagic transformation were absent outside the lesion area. Figure 1 shows that hemorrhage volume in rats treated with rtPA was significantly higher than in saline-treated animals. There was no difference in hemorrhage volume between MION-injected rtPA-treated rats and rtPA-treated rats that did not receive MION. In addition, validation of the spectrophotometric hemoglobin assay in presence of MION demonstrated that addition of MION had no effect on standard absorbance curves and therefore did not influence the quantification of intracerebral hemorrhage (data not shown).

Delayed rtPA treatment did not result in clear blood flow improvement, as evident from ROI analysis in areas with and without subsequent hemorrhage (Figure 2A). In addition, relative CBV was not significantly altered after rtPA treatment.
In accordance with earlier findings, we found that delayed rtPA therapy in a rat embolic stroke model led to increased hemorrhagic transformation in the ischemic territory. We have previously shown that intracerebral hemorrhage after rtPA treatment emerges in areas with early BBB damage. In the present study we describe acute secondary signal intensity reductions on susceptibility contrast-enhanced MR images during late rtPA delivery in ischemic animals that were preloaded with the contrast agent MION. Importantly, these changes in magnetic susceptibility and consequent local increase in $\Delta R_2^*$ involved regions with subsequent hemorrhagic transformation.

MION is an iron oxide–based superparamagnetic intravascular contrast agent with a blood half-life of several hours. Intravenous injection of MION resulted in a steady state signal intensity reduction on the susceptibility-weighted MR images throughout the entire brain. Thus, although flow levels were clearly lowered, residual perfusion allowed MION to be distributed in the ischemic territory. Administration of rtPA at 6 hours after MCA occlusion did not result in significant changes in relative CBF, and relative CBV, which is in agreement with our previous findings. Thus, the secondary change in $\Delta R_2^*$ that we detected during rtPA injection cannot be explained by a reperfusion-induced increased intravascular MION concentration. Moreover, the $\Delta R_2^*$ increase relative to pre-MION levels would correspond with high relative CBV values, beyond physiologically likely levels (>400% of contralateral). An alternative explanation would be that intracerebral hemorrhage causes this $\Delta R_2^*$ alteration. Extravasation of blood into tissue will lead to gradual deoxygenation of extravasally accumulated hemoglobin. Deoxyhemoglobin is paramagnetic and would induce a signal intensity reduction on susceptibility-weighted MRI. However, intracerebral hemorrhage during hyperacute stages is believed to consist primarily of oxyhemoglobin and consequently would be undetectable on susceptibility-weighted MRI. Moreover, earlier studies have shown that the core of a hyperacute hemorrhagic region is isointense or even hyperintense on T2*-weighted MR images, possibly because the T2* prolonging effect of extravasated fluid (see our previous study) is stronger than the T2* shortening effect of potentially present paramagnetic deoxyhemoglobin. Correspondingly, we did not detect signal intensity declines in rtPA-treated animals that did not receive MION. The rapid signal drop that we found in MION-injected animals during rtPA delivery is therefore not likely to be the result of acute deoxygenation of extravasated blood.

We hypothesize that the susceptibility-weighted signal intensity decrease is due to leakage of MION from the vasculature, accumulating in brain tissue during acute rtPA delivery. Increased BBB disturbance and widespread Gd-DTPA enhance-
ment on postcontrast T1-weighted MRI has been detected early after rtPA therapy. Recently, we have reported increased leakage of Gd-DTPA before rtPA administration in areas with hemorrhage after rtPA. MION, which has a much larger hydrodynamic diameter (approximately 20 nm) than Gd-DTPA (<1 nm), apparently did not leak out of the vasculature before rtPA therapy. Introduction of rtPA may seriously aggravate degradation of microvascular barriers in ischemic brain tissue. Microvascular barriers are mainly composed of a BBB and basal lamina. The BBB is formed by interendothelial tight junctions that allow selective transport, and the basal lamina is a structural barrier that prevents cellular blood elements from extravasating from the microvessels. Studies by Hamann et al suggest that hemorrhagic transformation is associated with breakdown of the basal lamina. Activation of the plasminogen-plasmin system by rtPA and release and activation of metalloproteinases have been suggested as important factors in the degradation of basal lamina components. In addition, thrombolysis-induced reperfusion could lead to injury to microvascular barriers, eg, through enhanced formation of reactive oxygen species and metalloproteinase activation. Inhibition of reactive oxygen species formation or metalloproteinase activation has been shown to reduce rtPA-associated hemorrhage in animal models. Our perfusion MRI results demonstrate that reperfusion was deficient early after rtPA administration. It should be mentioned, however, that our measurements were limited to 1 post-rtPA time point, thereby preventing the detection of potential hyperacute and transient reperfusion and/or delayed CBF increase.

Although some hemorrhage was present outside ischemic territories in the National Institute of Neurological Disorders and Stroke rtPA trial, our model only showed hemorrhage in the ischemic MCA zone. Severe ischemia may have already resulted in damage to the blood vessels per se. Although not statistically significant, perfusion was somewhat lower in areas with subsequent hemorrhagic transformation compared with nonhemorrhagic areas, which is in agreement with earlier studies. Still, on the basis of findings in our previous study and the present study, a consequential impairment of microvascular barriers would be relatively mild, merely allowing leakage of subnanometer structures. However, our present data clearly suggest that late thrombolytic treatment with rtPA accelerates breakdown of

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Figure 3. Susceptibility contrast-enhanced images of coronal rat brain slices in rtPA- (A, B) and saline-treated (D, E) rats. A and D, One minute before treatment. B and E, Thirty minutes after onset of treatment. Corresponding unstained brain sections are shown 3 hours after rtPA (C) or saline (F) treatment. White arrowheads depict the area with a large signal intensity drop on the susceptibility contrast-enhanced images early after rtPA administration (B), which corresponded with the regions with marked hemorrhagic transformation (C). Note that major signal intensity reductions and clear hemorrhagic transformation were absent in the striatum, which was also part of the ischemic lesion (black arrowheads).

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Figure 4. MION-induced ΔR2* on susceptibility contrast-enhanced images in areas of subsequent hemorrhagic transformation (HT) and in areas without HT (non-HT) at 1 minute before (white bars) and 30 minutes after (black bars) onset of rtPA or saline administration (mean±SEM). *P<0.05 vs before treatment. Since hemorrhagic transformation was absent or minor after saline treatments, HT areas could not be unequivocally identified in saline-treated animals. ROIs with HT were located in the cortex in 2 and in the subcortex in 4 of 6 rtPA-treated animals.

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Figure 5. A, Time course of ΔR2* changes during rtPA administration in regions with subsequent hemorrhagic transformation in rats that received MION before rtPA injection (n=6) (black line) and rats that did not receive MION (n=4) (gray line). Values are mean±SEM (error bars are displayed for every fifth data point). B, Individual rats that received MION. ROIs with HT were located in the cortex (n=2 of 6 MION-injected animals; n=1 of 4 non–MION-injected animals) or subcortex (n=4 of 6 MION-injected animals; n=3 of 4 non–MION-injected animals).
the BBB and basal lamina, resulting in subsequent hemorrhagic transformation. Although it is unclear at what stage the larger erythrocytes (diameter >3 μm) extravasate, clear intracerebral hemorrhage was evident as early as 2 to 3 hours after rtPA therapy but may evolve further over longer periods of time. Finally, it should be mentioned that ischemia-induced stasis of blood may have led to hemolysis. Hemolysates may contribute to parenchymal damage as well as enhance rtPA activity, which could have been a factor in the exacerbation of hemorrhagic transformation.

In conclusion, we have demonstrated that late rtPA treatment rapidly exacerbates early ischemia-induced BBB and basal lamina injury and leads to hemorrhagic transformation in our optimized experimental model. This process may involve direct interference of rtPA with the endothelial system and/or reperfusion injury (eg, oxidative stress). Despite its potential to reverse cerebral ischemia, our data emphasize that the therapeutic efficacy of rtPA, especially at later stages, can be seriously affected by enhanced breakdown of microvascular barriers and intracerebral hemorrhage.

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