Differences in Clot Preparation Determine Outcome of Recombinant Tissue Plasminogen Activator Treatment in Experimental Thromboembolic Stroke

Frank Niessen, MD; Thomas Hilger, PhD; Mathias Hoehn, PhD; Konstantin-A. Hossmann, MD, PhD

Background and Purpose—Thrombin-induced clots used in experimental thromboembolic stroke differ from clots forming spontaneously under clinical conditions. We investigated whether this difference influences the efficacy and outcome of thrombolytic treatment.

Methods—In rats, the middle cerebral artery was occluded by intracarotid injection of fibrin-rich clots, prepared either according to established methods by adding thrombin to freshly drawn arterial blood or by spontaneous coagulation. The mechanical properties of clots were determined in vitro by elasticity and plasticity tests. One hour after embolism, thrombolysis was started by intra-arterial application of recombinant tissue plasminogen activator (rtPA) (10 mg/kg). Treatment efficacy was monitored by MR measurements of blood perfusion, apparent diffusion coefficient (ADC), T2 relaxation time and blood-brain barrier permeability, and by pictorial measurements of ATP and pH.

Results—Thrombin-induced clots were classified as elastic, and spontaneously forming clots were classified as plastic. Middle cerebral artery embolism with thrombin-induced or spontaneously forming clots led to similar reduction of perfusion and ADC, but rtPA treatment efficacy differed greatly. In the spontaneously forming clot group, blood perfusion returned to or above control within 2 hours, ADC and ATP normalized, tissue pH exhibited alkalosis, and T2 and blood-brain barrier permeability did not change. In the thrombin-induced clot group, in contrast, blood reperfusion was delayed, ADC and ATP remained reduced, tissue pH was acidic, and edema developed, as reflected by increased T2 and blood-brain barrier permeability.

Conclusions—rtPA-induced thrombolysis promotes rapid reperfusion and tissue recovery in animals embolized with spontaneously forming clots but not in those embolized with thrombin-induced clots. This difference is explained by the different mechanical and possibly molecular consequences of clot preparation and must be considered for the interpretation of thrombolysis experiments. (Stroke. 2003;34:2019-2024.)

Key Words: brain edema ■ cerebral hemorrhage ■ stroke, experimental ■ thrombin ■ thrombolysis

Thrombin, an endopeptidase with important function in blood coagulation, has profound effects on virtually every aspect of vascular wall biology, including regulation of vessel tone, vascular permeability, smooth muscle cell proliferation, differentiation, migration, vascular development, atherogenesis, and angiogenesis.1 Thrombin is also neurotoxic, especially in combination with plasminogen activators.2

One of the main reasons for using thrombin-induced clots instead of spontaneously forming clots is the higher density of the fibrin meshwork and, as a consequence, the higher reproducibility of infarct size.5 However, with the advent of noninvasive imaging techniques, variability is a lesser concern because pretreatment control recordings are obtained in each individual animal. We therefore used a multiparametric MRI protocol to compare the effect of recombinant tissue plasminogen activator (rtPA) on thrombin-induced and spontaneously forming clots with respect to cerebral reperfusion, edema formation, blood-brain barrier permeability, and tissue survival.

Materials and Methods

Animal Model

All experiments were performed in accordance with the National Institutes of Health animal protection guidelines and were approved by the local government authorities.

Male Wistar rats weighing 320 to 400 g were anesthetized with 2% isoflurane in a 2:1 mixture of N2O/O2 and prepared for intraca-
rotid embolism and general physiological monitoring as previously described.6 Autologous, thrombin-induced, fibrin-rich blood clots were made as follows. Immediately after cannulation of the femoral artery, 0.05 mL fresh arterial blood was mixed with α-thrombin from rat plasma (30 U/mL Sigma T-5772, in 0.15 mL normal saline) and rapidly (2.5 mL/min) injected into PE-50 tubing. Three hours later, the tubing was cut into 5-cm-long pieces, and the clot material was removed by normal saline flushing. The clot was then washed twice in saline for 30 seconds and put into a solution of rat albumin (1 mg/mL) and phosphate-buffered saline. Fibrin-rich clot segments were selected and cut into cylindrical pieces approximately 0.35×1.5 mm in size. After 15 minutes, 4 fibrin-rich clots were drawn into an 80-cm-long PE-50 tube, which, in turn, was connected to the external carotid artery catheter. Autologous, spontaneously forming clots were prepared in the same way but without the addition of thrombin. Again, fibrin-rich segments were selected and cut into 4 pieces of the same diameter and length.

In 16 rats, experimental embolic stroke was induced inside the magnet by injecting 4 clots of either group over 30 seconds into the carotid circulation. Animals were randomly assigned to the thrombin-induced clot (TC) or spontaneously forming clot (SC) group. While the injection was performed, the right common carotid artery was temporarily occluded with a remotely controlled occlusion device. One hour after clot embolism, animals received ipsilateral intracarotid infusion of rtPA (10 mg/kg, Boehringer Ingelheim) dissolved in 2 mL distilled water. Ten percent of the solution was given as a bolus, and the rest was given as a constant infusion over 1 hour. Survival time after initiation of therapy was 7 hours.

Assessment of Mechanical Properties of Clots
Clots were differentiated into 2 classes with the use of the clot-bending test. Clots that returned after 90° bending to the former shape were classified as elastic, and clots that did not were classified as plastic. The mechanical properties of clots were furthermore evaluated by measuring the peak pressure required to pass saline-suspended clots at a flow rate of 1 mL/min through an 80-μm glass capillary.

Magnetic Resonance Imaging
Nuclear MR measurements were performed at 4.7 T in a Bruker BioSpec system (Bruker Medical) as described previously.5,6 An apparent diffusion coefficient (ADC) multislice set, a T2 relaxation–weighted multislice set, and a perfusion-weighted image (PWI) at the level of the caudate putamen were obtained before embolism and after each hour up to 7 hours after embolism. A 3-dimensional gradient-echo sequence with repetition time of 50 ms and echo time of 8.3 ms was used to detect blood-brain barrier disturbances. Measurements were performed before and 5 minutes after intravenous injection of the contrast agent gadolinium-DTPA (GdDTPA) (0.05 mL Magnevist).

The strongly T1-weighted sequence emphasizes regions with extravasated contrast agent. Areas of blood-brain barrier disturbance are detected by subtraction of a brain image before administration of the contrast agent.

Image Analysis
ADC and T2 maps and normalized PWIs were transferred to a Macintosh Power PC 7200/66(Apple) and submitted to image analysis with the use of the image processing software IMAGE (National Institutes of Health). Relative ADC, T2, and perfusion maps were calculated pixelwise by dividing ADC, T2, and perfusion maps by the corresponding preembolic data. The ischemic lesion was defined as the decline of ADC to <80% of control because this threshold correlates with the loss of ATP.8

ADC, T2, and PWI signal intensity were measured in the parietal cortex, which represents the center of the ischemic territory. Values were expressed as percentage of preischemic control. Successful reperfusion was defined as PWI signal returning to or above preischemic control value after ischemia.

### Results

#### General Physiological Observations
Twelve of 16 animals (n=6 in each group) submitted to intracarotid clot embolism were exposed to the full experimental protocol. The other experiments were terminated earlier: 1 experiment in each group because of respiratory failure and 1 experiment in each group because of uncontrollable bleeding from surgical wounds.

In animals with successful completion of the protocol, physiological variables (mean arterial blood pressure, heart rate, temperature, pH, PCO₂, PO₂, serum potassium, hematocrit fraction, and glucose) were within the normal range throughout the observation period, and there was no difference between the groups.

#### Mechanical Clot Properties
The difference of the mechanical clot properties was striking. All clots from the SC group were classified as plastic, while all clots from the TC group were elastic (see Materials and Methods). The difference in mechanical properties was also reflected by the peak pressure required to pass clots through the tip of an 80-μm capillary. Peak pressure was $61 \pm 46$ mm Hg in the SC group and $222 \pm 94$ mm Hg in the TC group ($P<0.01$). Clots of the SC group disintegrated after

<table>
<thead>
<tr>
<th>Incidence of Cerebral Hemorrhage</th>
<th>Histological Score</th>
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<tr>
<td>Group</td>
<td>0 1 2 3 4</td>
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<tr>
<td>Thrombin-induced clot (TC) (n=6)</td>
<td>4 2 0 0 0</td>
</tr>
<tr>
<td>Spontaneously forming clot (SC) (n=6)</td>
<td>4 1 1 0 0</td>
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Brain sections were graded for hemorrhage as follows: 0, no hemorrhage; 1, single microscopically visible hemorrhage; 2, multiple microscopically visible hematomas; 3, macroscopically visible non–space-occupying hemorrhage; 4, macroscopically visible space-occupying hemorrhage. The difference between SC and TC groups is statistically not significant (Mann-Whitney U test).

#### ATP and pH Imaging
Brains were frozen in situ at 7 hours after embolism, removed from the skull at −20°C, placed in a cold box, and sliced into 20-μm sections with a cryostat microtome. Coronal sections corresponding to the 6 levels of ADC slices were processed for the regional distribution of ATP by evoking substrate-specific bioluminescence. Regional tissue pH was measured in coronal sections adjacent to the ATP images with the use of umbelliferone fluorescence (for technical details, see Niessen et al6).

#### Histology
Cryostat sections were stained with hematoxylin and eosin and inspected by light microscopy (Leica MZFL.I11 microscope) for intracerebral hematomas. The severity of hemorrhages in the ischemic territory was scaled as described in the Table.

#### Statistical Analysis
All data are presented as mean±SD. Physiological variables, MR data, and the incidence of cerebral hemorrhages were compared with the Mann-Whitney U test; differences were considered significant at $P<0.05$. Measurements were performed by a person blinded to the experimental groups. Statistical analysis was performed with the use of the software package StatView for Windows (release 5.0.1, SAS Institute, Inc.).
being forced through the capillary, while clots from the TC group returned to their initial shape. These measurements demonstrate that clots from the SC group are less elastic than those produced by the addition of thrombin.

Perfusion-Weighted Imaging
Before clot embolism, PWI exhibited symmetrical distribution of signal intensity in both hemispheres. Infusion of clots into the right carotid artery led to a marked decline of PWI signal intensity in the ipsilateral cortex (Figure 1). Immediately after embolization, the PWI signal decreased to 50±13% and 45±7% of control for the SC and TC groups, respectively (no significant difference between the 2 groups; Figure 2). During the 1-hour interval between embolization and the beginning of treatment, the PWI signal increased to 61±17% in the SC group and to 50±11% in the TC group (no significant group difference). Within 2 hours after start of rtPA treatment, PWI signal recovered to the preembolic level in the SC group, but it did not change in the TC group (102±24% versus 51±16%; P<0.05). While the SC group developed slight hyperperfusion (106±20%) between 2 and 4 hours after the start of rtPA treatment, PWI in the TC group returned to only 80±34% of control between 4 and 5 hours after the start of rtPA treatment. In the SC group, the time to reperfusion, defined as the interval from the start of treatment to the return of PWI signal intensity to control level, was 1.6±0.8 hours. In the TC group, PWI signal intensity returned to control in only 2 of 6 animals between 4 and 5 hours after onset of treatment.

ADC Imaging
Preembolic ADC maps did not exhibit asymmetries between the 2 hemispheres (Figure 1). Embolism led to a reduction of ADC in the ipsilateral hemisphere, predominantly in the cortical middle cerebral artery (MCA) territory. At the start of treatment 1 hour after MCA embolism, the ADC lesion volumes were not significantly different, with 17.4±8.2% and 20.8±13.0% of ipsilateral hemisphere for the SC and TC groups, respectively. Six hours after treatment, ADC lesion volume decreased in the SC group to 13.6±6.1%, whereas it increased to 24.8±14.1% in the TC group.

T2 Imaging and Evaluation of Blood-Brain Barrier Disturbance
The temporal evolution of the T2 relaxation time is presented in Figures 1 and 2. Directly after embolization, ADC decreased to 85±8% and 79±11% in the SC and TC groups, respectively. The 1-hour pretreatment period, it decreased further to 82±10% and 72±6%, respectively (no significant group difference). At 2 hours after start of rtPA treatment, the ADC of the SC group recovered to preembolic values, while in the TC group ADC did not improve (96±6% versus 69±10%; P<0.01). In the latter group, ADC exhibited no further changes and remained well below 80% until the end of the experiment.
tion, and at 6 hours after the onset of rtPA treatment it reached 122±5%, compared with 102±3% in the SC group (P<0.05).

Disturbances of the blood-brain barrier were assessed by intravenous GdDTPA application. In the SC group GdDTPA extravasations were present in all animals (Figure 1).

Biochemical Imaging and Histology
In the SC group, ATP values at the end of the observation period were normal, and pH exhibited reactive alkalosis. In contrast, energy metabolism was severely disturbed in the TC group, as reflected by the loss of ATP and tissue acidosis (Figure 1).

Hematoxylin-eosin–stained brain sections were investigated for the occurrence of cerebral hemorrhages (Table). Surprisingly, minor hemorrhages were detected in only 2 animals of each group, despite the high incidence of blood-brain barrier disturbances after TC embolization.

Discussion
rtPA treatment of experimental thromboembolic stroke leads to a greatly different outcome, depending on the preparation of clots used for MCA embolization. Previous investigations demonstrated differences in clot lysability depending on erythrocyte and platelet content, but the influence of thrombin has not been studied previously. Obviously, endogenous thrombin is also involved in the spontaneous clotting of blood, but when exogenous thrombin is added, outcome of thrombolysis is greatly impaired. This difference was not due to spontaneous thrombolysis in the SC-embolized animals because before treatment, 1 hour after embolism, both groups exhibited the same reduction of blood flow and ADC impairment. Because other pretreatment variables were also similar in both groups, the only plausible reason for the different outcome was the higher concentration of thrombin in the clots of the TC group. Thrombin may interfere with rtPA treatment in 2 ways: indirectly by modifying the mechanical properties of clots and directly by its molecular effect on the vulnerability of brain tissue. In the following, these effects will be discussed separately.

Mechanical Consequences
The reversal of ischemic injury by rtPA treatment, as assessed by MRI of the ADC and the bioluminescence imaging of ATP, was clearly related to the speed of reperfusion of the ischemic territory. In the SC group, blood flow increased above control within 2 hours after the onset of treatment (postischemic hyperperfusion), indicating rapid recanalization in combination with postischemic vasodilation. In the TC group, in contrast, recanalization was greatly delayed, as reflected by the late and incomplete reversal of blood flow. The relationship between the efficacy of early reperfusion and functional recovery has been documented amply in various models of brain ischemia and confirms that the chances of postischemic recovery decline with declining speed of flow restitution.

This effect is not due solely to the prolongation of the ischemia time when recirculation is delayed. The main reason is the inverse relationship between the speed of metabolic recovery and the generation of cytotoxic brain swelling. When blood flow slowly increases, anaerobic glycolysis is resumed before oxidative metabolism begins to recover. As a result, lactacidosis further progresses, and the restoration of
energy-dependent ion exchange pumps is delayed, both of which favor the generation of postischemic cell swelling. As postischemic swelling increases vascular resistance and reduces the local blood perfusion pressure, a vicious circle is generated that eventually may result in permanent flow arrest and tissue necrosis.10

The present investigation suggests that thrombolysis and hence the speed of reperfusion depend on the mechanical properties of clots, which, in turn, are dependent on clot thrombin content. In the TC group, 1 mL clot suspension was enriched with 6.4 NIH U thrombin, which almost doubled the thrombin activity compared with the SC group.11 Thrombin activates factor XIII, which is involved in cross-linking the fibrin network,12 and with increasing thrombin activity, the diameter of fibrin filaments decreases, leading to a denser and stiffer fibrin meshwork.13 The tighter fibrin meshwork influences the lysability of clots because the smaller pores between the more tightly packed fibrin filaments build up a higher resistance to blood plasma and the penetration of rtPA. As a result, the speed of thrombolysis declines, which is in full agreement with our observation of the much longer time to reperfusion in thrombin-induced clots.

Different fibrin conformation may also be the reason for the previously observed difference in lysis times of human blood clots with similar light microscopical appearance.14 In fact, since the difference in the density of the fibrin meshwork can only be detected at an ultrastructural level, the histological similarity does not exclude conformational differences.

Molecular Consequences
Fibrin-rich clots contain a reservoir of enzymatically active thrombin,15 which is released from clot material during thrombolysis and may have secondary effects, such as enhancement of platelet procoagulant activity, promotion of edema formation, induction of vasospasm, and possibly neurotoxicity. It is conceivable that such secondary effects also contribute to the observed differences in outcome between the 2 groups.

Procoagulant Activity
Thrombin released from the lysed clots may potentiate the procoagulant activity of platelets15 and could lead to vascular reocclusion. It is conceivable that this effect is enhanced by the addition of thrombin to the coagulating blood. Conversely, activated protein C, which inhibits the activation of prothrombin, reduces the relative mass of fibrin within thrombi16 and thereby reduces microvascular obstructions.17 Interestingly, small amounts of thrombin may be protective by increasing activated protein C, a phenomenon referred to as a thrombin paradox.18 The risk of postischemic reocclusion is therefore difficult to predict.

Brain Edema and Blood-Brain Barrier Disturbances
Direct infusion of 10 or 100 U thrombin in 10 µL solution into the basal ganglia of rat results in cerebral edema.19 Thrombin released from the lysed clot may therefore contribute to the breakdown of the blood-brain barrier and the formation of vasogenic edema, as described in this and an earlier communication.20 However, edema is also a function of the severity of ischemic injury, which was more pronounced in the TC group. A distinction between the 2 pathomechanisms is therefore not possible in our experimental setting.

Interestingly, the disturbed blood-brain barrier permeability in the TC group was not associated with hemorrhagic transformations, as recently described in our and another laboratory.21,22 However, in the present study brains were examined already at 7 hours after the onset of treatment compared with 3 days in our earlier investigation. Hemorrhagic transformation therefore seems to be a rather late consequence of thrombolytic treatment.

Vasoconstriction
Another factor contributing to the incomplete reperfusion in the TC group may be vasoconstriction. Although thrombin initially induces vasorelaxation, this effect may be followed by persistent dose-dependent vasoconstriction.23 Obviously, the dominant cause for the delayed reperfusion in the TC group is the stiffer consistency of the clots, but the absence of postischemic hyperemia once the clot was lysed may be due to a late vasoconstrictive effect.

Neurotoxicity
Evidence from several studies suggests that thrombin and its receptor may be involved in neurodegenerative processes associated with brain injury or brain ischemia (for review, see Turgeon and Houenou24). However, in vitro data obtained in hippocampal cultures submitted to oxygen-glucose deprivation demonstrate that thrombin may have greatly different effects, depending on its concentration in the culture medium:25 at 0.01 U/mL it is clearly neuroprotective, at 0.1 to 1.0 U/mL it becomes neutral, and at 10 U/mL it replicates the previously observed neurodegenerative effects. In the present study thrombin-induced clots were formed with 30 U/mL thrombin, but the local concentration at the embolization site is obviously much lower. It is therefore difficult to predict whether the higher amount of thrombin released from clots in the TC group reaches neurotoxic levels.

Conclusions
Our investigation demonstrates a marked difference in the outcome of rtPA treatment of thromboembolic stroke, depending on the preparation of clots used for vascular embolization. The faster and functionally much more efficient thrombolysis of spontaneously forming clots compared with the experimentally more widely used thrombin-induced clots is explained by the mechanical and possibly molecular consequences of clot preparation and must be considered for the interpretation of thrombolysis experiments.

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