Comparison of TNK With Wild-Type Tissue Plasminogen Activator in a Rabbit Embolic Stroke Model

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Background and Purpose—Tissue plasminogen activator (tPA) is an effective treatment for stroke, but its utility is limited by fear of cerebral hemorrhage. Tenecteplase (TNK), a genetically modified form of wild-type tPA, exhibits a longer biological half-life and greater fibrin specificity, features that could lead to fewer cerebral hemorrhages than wild-type tPA in stroke patients.

Methods—We injected radiolabeled blood clots into the cerebral circulation of New Zealand White rabbits. One hour later, we administered tPA (n=57), 0.6 mg/kg TNK (n=43), 1.5 mg/kg TNK (n=27), or vehicle control (n=37). A blinded observer examined the brains for macroscopic hemorrhage using a semiquantitative score. We estimated thrombolysis by assessing the amount of radiolabel remaining in the cerebral vessels postmortem.

Results—Both wild-type tPA and TNK caused thrombolysis in most subjects. Hemorrhage was detected in 26% (6/23) of the control group, 66% (27/41) of the wild-type tPA group, 55% (16/29) in the 0.6-mg/kg TNK group, and 53% (9/17) in the 1.5-mg/kg TNK group (P<0.05, χ² test). The tPA group was statistically significantly different from the control group, but the TNK and tPA groups did not differ from each other. Neither TNK nor tPA affected the size of the hemorrhages.

Conclusions—TNK shows comparable rates of recanalization compared with wild-type tPA in a model of embolic stroke. While tPA increases hemorrhage rate, the hemorrhage associated with TNK treatment is not statistically different compared with controls or the tPA group. These findings suggest that TNK shows promise as an alternative thrombolytic treatment for stroke, but we could not demonstrate improved safety compared with wild-type tPA. (Stroke. 2001;32:748-752.)

Key Words: cerebral embolism ▪ hematoma ▪ hemorrhage ▪ thrombolysis ▪ thrombolytic therapy ▪ rabbits

Wild-type tissue plasminogen activator (tPA) is an effective thrombolytic therapy for stroke patients, despite an increased rate of cerebral hemorrhage.1,2 Tenecteplase (TNK), a genetically modified form of wild-type tPA, which seems to produce more rapid and complete thrombolysis. TNK has a longer biological half-life and is 14 times more fibrin specific than tPA.3 The longer half-life of TNK allows bolus administration, rather than the prolonged infusion required by wild-type tPA. The increased fibrin specificity of TNK could reduce the potential for hemorrhage, as was demonstrated in human myocardial infarction trials.4 With tPA therapy, the systemic activation of plasminogen and increased plasmin generation that follow treatment was associated with peripheral and cerebral hemorrhages in the treatment of myocardial infarction.5 In contrast to this, TNK exerts its thrombolytic effect in the presence of high fibrin concentrations, such as occur within a blood clot, rather than in the systemic circulation where there is an absence of clotted plasma.6

TNK has been used in clinical trials for patients with myocardial infarction.7-8 When TNK and tPA were compared in clinical trials, there was no difference in the incidence of cerebral hemorrhage between the 2 treatments. In addition, TNK appeared to avoid the “plasminogen steal” effect, which is a paradoxical decrease in tPA effectiveness seen when plasminogen leaches from the clot in the presence of high tPA concentrations.9 Patients given a 5- to 10-second intravenous bolus of TNK had plasma levels comparable to those treated with a 90-minute infusion of tPA, at time points up to 3 hours after administration.7 TNK also achieved 90-minute grade 3 flow according to the Thrombolysis in Myocardial Infarction (TIMI) classification, similar to that of a 90-minute infusion of tPA.7 Unlike tPA, TNK was found to be devoid of procoagulant effects in myocardial infarction patients.10

Animal studies also demonstrated that TNK might be a superior therapy to tPA. For example, in a rabbit model similar to that presented in our study, TNK-treated animals showed a lower cerebral hemorrhage rate than control-treated animals.9 TNK was also 3 times more potent at lysing clots than tPA in a dog model of arterial and venous thrombosis.11 The benefits seen in the myocardial infarction patients treated with TNK, plus the encouraging results from animal studies, suggested that TNK might prove to be a safer and more effective...
therapy than tPA in stroke patients. We tested this hypothesis using a rabbit model of thromboembolic stroke.12,13

Materials and Methods

The methods were modified from our previous protocol, described in detail elsewhere.14 Briefly, 164 male New Zealand White rabbits (weight, 2 to 3 kg) were anesthetized with halothane. We inserted a plastic catheter oriented toward the brain into the right common carotid artery of each subject, after ligating the external carotid artery and all visible branches of the common and internal carotid arteries. We closed the incision around the catheter so that the distal end was readily accessible. We filled the catheter with 2 mL of heparinized saline (33 U/mL) and plugged it with an injection cap. The animals recovered from anesthesia for at least 2 hours before embolization. Each rabbit was examined before embolization to ascertain that no complications had resulted from the catheterization surgery; abnormal animals were excluded. Emboli were formed by mixing donor arterial blood with a trace quantity of 51-Co-labeled 25-μm-diameter plastic microspheres. After 3 hours, the clot was sliced into small cubes weighing approximately 3 to 4 mg, which were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin. The amount of radiolabel present in each cube was measured with a gamma counter. We assume that the clots used in our model were fibrin-rich because they were composed of whole arterial blood allowed to clot for only 3 hours; the identical method of clot preparation was used across all treatment groups. To cause a stroke, one of the clot cubes was suspended in 3 mL saline and injected rapidly into the catheter, followed immediately by a 3-mL flush. Care was taken to ensure that no air bubbles were present in the catheter or syringe. If the animal did not react behaviorally (nystagmus, hemiparesis, or uncoordinated movements) within 3 minutes, another blood clot was injected in the same way. If there was no reaction after 2 clots, no further emboli were injected. After the embolization process was completed, we ligated the catheter close to the skin and cut off the rest of the catheter and injection port.

Animals that died before euthanasia were included in this study; the brains were fixed and sectioned as below. The surviving animals were euthanized (Beuthanasia-D Schering Plow Animal Health Care) 48 hours after embolization, and the brains were immersion fixed in 4% paraformaldehyde for at least a week to allow removal of the pial surface vessels and brain sectioning. A blinded observer evaluated the ventral surface of each specimen for the presence of visible clot in the right middle cerebral artery. Using microforceps, pial vessels were stripped off the embolized hemisphere and placed in a plastic tube for gamma counting. Then, the examiner sliced the brain into 5-mm coronal slices with a calibrated sectioning device and noted the presence, location, and type of each hemorrhage in each block. The number of hemorrhages was estimated by counting the number of section faces where hemorrhage was present, a semiquantitative method used in prior studies.13,15,16 Each brain slice has 2 “faces.” A hemorrhage can be embedded in the relatively thick block, showing only 1 face but not on the face of the adjacent block; therefore, the total number of faces per hemorrhage can be odd or even. This visual method of estimating the cerebral hemorrhage size correlates well with spectrophotometric analysis of hemoglobin content in hemorrhaged mouse brains.17 Since the hemorrhagic lesions tend to be heterogeneous, without discrete borders, microscopic quantification methods may be less accurate.14 Three types of hemorrhage were identified: (1) hemorrhagic infarction or red speckling of an area, usually surrounded by soft infarcted tissue; (2) punctate hemorrhages or isolated small red marks within the tissue; and (3) parenchymatous intracerebral hemorrhages, a large homogeneous mass of blood within the tissue. Next, the total radioactivity in the brains was measured by placing all the coronal blocks into plastic tubes for gamma counting. If <10% of the originally injected counts were found in the brain and pial vessels, we concluded that the labeled clot had not reached the brain. The data from these animals were excluded from further analysis, resulting in some imbalances in group sizes. Additionally, if no counts were found in the vessels, but a visible clot was noted in the middle cerebral artery, we rejected the subject, reasoning that the visible blood clot must have come from a source other than the prepared embolus. We defined thrombolysis by comparing the amount of radiolabel present in the vessels to that present in the whole brain. If we found <20% of the recovered radiolabel in the blood vessels, we concluded that the clot had been lysed, causing the majority of the microspheres to leave the embolus, becoming trapped in brain capillaries.

Drug Administration

We randomly allocated animals to treatment groups before the embolization procedure. Group sizes were based on previous experience, allowing for loss due to preparation difficulties (catheter failure or anesthetic complication). In addition, we excluded any subject in which <10% of the radiolabel was recovered postmortem (embolism failure). Throughout the experiment, we added subjects to replace those lost because of catheter or embolism failures, resulting in unequal group sizes. We included different treatment groups during each day, so that any variation would be spread across all treatment groups and over the entire duration of the experiment. We administered the treatments 1 hour after embolization: tPA (n = 57), low-dose TNK (n = 43), high-dose TNK (n = 27), and vehicle (n = 37). Additional rabbits without catheterization or embolization were given tPA (n = 5) or vehicle (n = 5) to ascertain whether tPA causes spontaneous cerebral hemorrhages. We chose 1.5 mg/kg as the high dose for TNK because it has been shown to produce >80% recanalization in a dose-response study using a rabbit model of carotid artery thrombosis.18 We chose 0.6 mg/kg as the low dose for TNK because that dose has previously been shown to produce complete clot lysis over 2 hours, while decreasing the hemorrhage rate, in a model similar to our model.3 We administered TNK at a constant rate over 3 minutes. We chose to administer 3.3 mg/kg wild-type tPA as a 20% bolus injection over 1 minute, with the remainder infused over 30 minutes.13,15,19 All drugs were supplied by Genentech, Inc. We analyzed the data with the χ2 test corrected for multiple comparisons using the Bonferroni technique and ANOVA when relevant.

All animal use procedures are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Animals and are approved by the animal care committee of the San Diego Veterans Administration Medical Center.

Results

Embolism Rate

The 2-embolus method was devised to increase the rate of successful embolization over the previous 1-embolus technique. Of 164 embolized rabbits, we found 10% of the label present in 110 (67%) of the subjects. The embolism failures were distributed among the groups as follows: control (n=14/37; 38%), tPA (n=16/57; 28%), TNK 0.6 mg/kg (n=14/43; 33%), and TNK 1.5 mg/kg (n=10/27; 37%). The success rate corresponds well with other experiments involving this model. Nonetheless, large numbers of rabbits were needed for this study to compensate for the relative inefficiency of the model.

Hemorrhage Rate

Figure 1 compares the hemorrhage rate between drug-treated and control-treated rabbits. In the control group, 26% (n=6/23) of the rabbits had cerebral hemorrhage present. The low dose of TNK caused hemorrhage in 55% (n=16/29) of the subjects, whereas the high dose of TNK had an associated hemorrhage rate of 53% (n=9/17). In the 5 unoperated rabbits treated with intravenous tPA, there was no sign of hemorrhage. Overall, there was a statistically significant difference in hemorrhage rates among the 4 treatment groups ($\chi^2=9.45$, $P<0.05$). When we compared individual treatment groups, we found the following: (1) tPA caused significantly more hemorrhages than the control treatment ($P<0.01$, $\chi^2$ test); (2) there was no significant difference in hemorrhage rate among the 2 TNK groups and the tPA-treated rabbits ($P>0.05$, $\chi^2$ test); and (3) the hemorrhage rates among the TNK groups and the control groups were not significantly different ($P>0.05$, $\chi^2$ test). Therefore, even though the TNK hemorrhage rates are nominally less that that for tPA, we do not conclude that TNK caused fewer hemorrhages.

Hemorrhage Size

Hemorrhage sizes are reported in Figure 2. There were no statistically significant differences among any of the study groups, indicating that none of the treatments exacerbated the size of hemorrhage compared with those found in the control rabbits. The size of hemorrhage in the 1.5-mg/kg TNK group was nominally lowest, but this difference was not significant. The Table illustrates the types of hemorrhage present in the different treatment groups. The majority of the hemorrhages observed were hemorrhagic infarction, but intracerebral hemorrhages and punctate hemorrhages were also present in each of the groups. Some of the animals had >1 type of hemorrhage. Hemorrhages occurred in the caudate, thalamus, temporal lobe, hippocampus, occipital and parietal cortex, hypothalamus, suprachiasmatic area, cerebellum, frontal lobe, pons, and midbrain. There were no differences in the distribution of types or location of hemorrhages among the treatment groups.

Thrombolysis Rate

Figure 3 compares thrombolysis among the treatment groups. All of the thrombolytic agents caused a significant increase in thrombolysis compared with the control treatment, in which 9% of the emboli were recanalized. In the tPA-treated group, 44% of the clots were lysed ($P<0.01$ after correction, $\chi^2$ test). Each TNK dose showed significant thrombolysis ($P<0.05$ after correction, $\chi^2$ test).

Discussion

We found that TNK caused hemorrhage after embolic stroke about as often as wild-type tPA (Figure 1). Hemorrhagic
infarction was the most common type of hemorrhage, the remainder being intracerebral hemorrhages and punctate hemorrhages (Table). None of the thrombolytics increased hemorrhage size compared with hemorrhages in the control group (Figure 2). Both wild-type tPA and TNK effected similar rates of thrombolysis (Figure 3). These data suggest that TNK is a safe and effective thrombolytic in experimental embolic stroke. The hemorrhage rates in the TNK groups were nominally less that of the tPA group and were not statistically significantly different than control hemorrhage rates. The tPA and TNK hemorrhage rates, however, were not statistically significant, meaning that the TNK hemorrhage rates are in between tPA and control and could be higher than control. The most rigorous interpretation of this result is that TNK and tPA exhibit similar hemorrhage rates, but larger trials could show that TNK causes fewer hemorrhages. Others have shown, using fewer subjects, that TNK causes fewer hemorrhages.3

We also sought to estimate the risk of inadvertently treating stroke mimics that are not ischemic. We included control groups to ascertain whether tPA alone might cause spontaneous hemorrhages: none of the rabbits that received tPA without undergoing catheterization or embolization suffered any hemorrhage. In addition, we examined the hemorrhage rate in rabbits who had failed the criteria for successful embolization (embolism failures) and showed no behavioral signs after injecting the blood clots (ie, negative controls). In those treated with TNK or tPA, we found no spontaneous cerebral hemorrhages either. These data suggest that giving thrombolytic treatment to subjects with stroke mimics, ie, with no ongoing cerebral ischemia, could be safe. This is an important finding because many stroke patients do not receive thrombolytic therapy because of difficulties in diagnosing stroke symptoms and fears of administering thrombolytics to a person for whom it would have no clinical benefit.21 It is reassuring to note the lack of hemorrhages in any subjects who suffered no ischemia.

It is difficult to compare hemorrhage rates between this and past studies because we modified the previous protocol to include a second embolus. We attempted to reduce the embolism failure rate, and thus improve the efficiency of the model, by injecting a second embolus if the first one failed. The 2-embolus method could lead to different rates of hemorrhage and thrombolysis compared with prior studies. Additionally, the doses of tPA investigated previously have varied greatly, from 3 to 10 mg/kg, and the infusions varied from 30 minutes to 2 hours, starting between 15 minutes before and 24 hours after embolization.15 The control hemorrhage rate of 26% corresponds relatively well to previous studies.22 However, our study showed a high incidence of hemorrhage in the tPA-treated group, which, unlike previous reports, proved to be significantly greater than the control group.12,16,20 The tPA-induced hemorrhage rate in clinical practice is also much lower than that in our experiment, but this could result because only symptomatic hemorrhages are identified in clinical trials. If asymptomatic hemorrhages are identified by CT scans, then the incidence of hemorrhagic transformation after embolic stroke is between 40% and 60%;23,24 This rate is closer to that seen in our experiment. Of note, our scoring method detects even smaller hemorrhages than can be seen with CT. If we had not included punctate hemorrhages in our calculations, the hemorrhage rates would have been much lower.

Using a similar model, Thomas et al3 found that 0.6 mg/kg TNK caused a lower hemorrhage rate than tPA. However, important differences in methodology may account for the differences in their findings compared with this study. Those studies were conducted under anesthesia, which alters the hemorrhage rate by affecting blood pressure responses.15 In the present study the rabbits were allowed to recover from anesthesia for at least 2 hours before the embolization. In addition, the use of 2 emboli in some animals may have caused a different hemorrhage rate. Finally, variations in drug potency from lot to lot are possible, but since TNK is now in clinical use, production methods are standardized (oral communication, Genentech, Inc, 1999). The lot we used in this study came from the clinical production line.

Our finding that TNK and tPA have similar thrombolytic activities in the rabbit stroke model indicates that fibrin specificity may be less relevant in thrombolytic therapy than had previously been thought. TNK was designed to be more efficacious as a thrombolytic and to have a lower risk of cerebral hemorrhage. For example, TNK has a longer half-life and slower plasma clearance rate than tPA in humans, dogs, and rabbits.11,25 Various animal studies suggested that the fibrin specificity of TNK would increase its thrombolytic activity compared with tPA.6,11 Since TNK has higher fibrin specificity than tPA, the hemorrhage rate could be lower.18,26–28 At variance with this prediction, we found that the hemorrhage rate of TNK was not statistically significantly less than that of tPA, despite being nominally lower.

We investigated 2 doses of TNK, chosen on the basis of thrombolytic efficacy and hemorrhage rate experiments con-
ducted previously. There was no significant difference in thrombolytic rate between the 2 TNK doses. This contrasts with previous findings: 1.5 mg/kg was the most effective in a range of TNK doses. However, this study does not indicate whether the difference between 1.5 mg/kg and the next lowest dose (0.75 mg/kg) was significant. It is possible that TNK is already acting at maximum efficacy in the range of 0.6 to 1.5 mg/kg. Alternatively, it could be that a 2-fold increase in dose (0.6 to 1.5 mg/kg) is not sufficient to see an increase in efficacy. In support of this theory, in a rabbit model similar to our model, there is an apparent difference in thrombolysis between 0.03 and 0.6 mg/kg but not between 0.3 and 0.6 mg/kg.3

To summarize, our study shows that there are no statistically significant differences in the thrombolytic efficacy or hemorrhage rates associated with TNK and tPA. Given the easier use of bolus administration, TNK could be a promising thrombolytic agent. Further pharmacological studies, including human trials, are required before concluding that TNK is a safer alternative to wild-type tPA.

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References


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