J Neurosurg 105:424–429, 2006

Lysis of intracerebral hematoma with stereotactically implanted tissue plasminogen activator polymers in a rabbit model

QUOC-ANH THAI, M.D., GUSTAVO PRADILLA, M.D., FEDERICO G. LEGNANI, M.D., RYAN M. KRETZER, M.D., WESLEY HSU, M.D., AND RAFAEL J. TAMARGO, M.D.

Division of Cerebrovascular Neurosurgery, Department of Neurosurgery, School of Medicine, The Johns Hopkins University, Baltimore, Maryland

Object. Currently no adequate surgical treatment exists for spontaneous intracerebral hemorrhage (ICH). Implantable polymers can be used effectively to deliver therapeutic agents to the local site of the pathological process, thus reducing adverse systemic effects. The authors report the use of stereotactically implanted polymers loaded with tissue plasminogen activator (tPA) to induce lysis of ICH in a rabbit model.

Methods. Ethylene vinyl acetate (EVAc) polymers were loaded with bovine serum albumin (BSA) only or with BSA plus tPA. In vitro pharmacokinetic (three polymers) and thrombolysis (12 polymers) studies were performed. For the in vivo study, 12 rabbits were fixed in a stereotactic frame, and 0.2 ml of clotted autologous blood was injected into the right frontal lobe parenchyma. After 20 minutes, control BSA polymers were stereotactically implanted at the hemorrhage site in six rabbits, and experimental BSA plus tPA polymers were implanted in six rabbits. Animals were killed at 3 days, and blood clot volume was assessed.

The pharmacokinetic study showed release of 146 ng of tPA over 3 days. The tPA activity correlated with in vitro thrombolysis. In the in vivo study, the six animals treated with tPA polymers had a mean ($\pm$ standard error of the mean [SEM]) thrombus volume of 1.43 ± 0.29 mm^3 at 3 days, whereas the six animals treated with blank (BSA-only) polymers had a mean ($\pm$ SEM) thrombus volume of 19.99 ± 3.74 mm^3 ($p < 0.001$).

Conclusions. Ethylene vinyl acetate polymers release tPA over the course of 3 days. Stereotactic implantation of tPA-loaded EVAc polymers significantly reduced ICH volume. Polymers loaded with tPA may be useful clinically for lysis of ICH without the side effects of systemic administration of tPA.

KEY WORDS • intracerebral hemorrhage • intracerebral hematoma • tissue plasminogen activator • controlled-release polymer • stereotactic targeting • rabbit

STROKE caused by spontaneous ICH affects more than 37,000 people each year in the US and accounts for about 15% of all strokes.[4,6,9,12,16] Although the incidence of ICH is lower than that of ischemic stroke, ICH is associated with higher mortality and morbidity than any other form of stroke. The damage from ICH is mainly due to acute local mass effect, focal damage to adjacent tissues, and poorly understood subacute mechanisms that continue to injure the surrounding viable brain tissue.

Currently there are no effective medical[1,14,24,27] or surgical[2,3,8,11,13,28] treatments for ICH, but thrombolytic agents may be useful in its management.[12,23] Previous clinical studies have reported that ICH volume is decreased by instilling urokinase by means of a stereotactically implanted catheter followed by aspiration of the liquefied thrombus.[12,23] Tissue plasminogen activator is another thrombolytic agent that is effective and approved for intraarterial thrombolysis, but it is contraindicated in the presence of ICH due to the systemic side effects. Systemic administration of tPA leads to cleavage of serum plasminogen into plasmin, which results in thrombolysis and can increase the risk of repeated bleeding.

Controlled-release polymers[19,20] implanted locally can be used to deliver tPA directly at the site of intracerebral hemorrhage. Because ICH, unlike other hemorrhagic strokes, is predominantly a focal pathological process, the mass effect occurs around the hemorrhagic center. Controlled-release polymers provide a potentially efficacious method of delivering high drug doses directly to the site of hemorrhage without systemic side effects. The local delivery of therapeutic drugs also has several theoretical advantages, including bypassing the blood–brain barrier, which prevents entry of most drugs into the CNS. We have demonstrated that local or regional drug delivery using controlled-release polymers is a preferred strategy when treating certain CNS conditions.[19,21,22]

We report the formulation of a tPA polymer that has pre-

Abbreviations used in this paper: BSA = bovine serum albumin; CNS = central nervous system; EDTA = ethylenediamine tetraacetic acid; EVAc = ethylene vinyl acetate; ICH = intracerebral hemorrhage; PBS = phosphate-buffered saline; SEM = standard error of the mean; tPA = tissue plasminogen activator.

dictable release pharmacokinetics in vitro and describe the stereotactic implantation of tPA polymers in vivo, the toxicity associated with tPA polymers, and the resulting thrombolysis in a rabbit model of ICH.

Materials and Methods

Experimental Design

Four sets of experiments were conducted to evaluate in vitro pharmacokinetics, in vitro thrombolysis, in vivo toxicity, and in vivo thrombolysis.

In the in vitro pharmacokinetics study, release kinetics of tPA polymers were evaluated by suspending three tPA polymers in a buffer solution. At 1, 3, 6, 9, 12, 24, 48, and 72 hours, the polymers were moved to a new container of buffer solution, and then the concentration of tPA released in each buffer solution container was determined.

The in vitro thrombolysis study included four groups of three polymers each. The thrombolytic activity of three blank polymers, three polymers containing BSA, three containing tPA, and three containing tPA plus BSA was studied by placing the polymers in the center of a Petri dish filled with clotted rabbit blood. At 24, 48, and 72 hours, the cross-sectional area of thrombolysis was measured.

In the in vivo toxicity study, there were two groups of three animals each. One group underwent intracerebral implantation of tPA polymers, and the other group received polymers containing BSA. Animals were evaluated for 12 weeks and then killed. Tissue samples from various organs were processed for gross and histological pathological examination.

In the in vivo thrombolysis study, 15 animals were randomly assigned to three experimental groups. In the first group, six animals received an ICH followed by implantation of a 0.02% tPA/EVAc polymer (weight 10 mg, 2 μg of tPA). In the second group, six animals received an ICH followed by implantation of an empty EVAc polymer. Polymer implantations were performed 20 minutes after induction of ICH. In a third group of three animals, a sham surgery was performed without ICH or polymer implantation. Rabbits were killed at 3 days, and the ICH volume was calculated for each group.

Polymer Preparation

We have previously described the technique for incorporating various drugs into controlled-release EVAc polymers.[21,25,26] Ethylene vinyl acetate polymer (40% vinyl acetate by weight [Elvax 40-W E4902-30 V51212; DuPont, Wilmington, DE]), 98% BSA (pH 7) (Sigma Chemical Co., St. Louis, MO), and single-chain tPA from human melanoma cells (Sigma Chemical Co.) were dissolved in methylene chloride (Fisher Chemicals, Fair Lawn, NJ), as previously described.[18] Polymers were formulated as 0.02% tPA/EVAc (weight/weight), 20% BSA/EVAc, and 0.02% tPA plus 20% BSA/EVAc. Then, 1 ml of the solution was pipetted into cylindrical glass molds (5 × 27 mm) at −70°C. The frozen mixture was extracted from the glass molds with a cold spatula. Methylene chloride was allowed to evaporate for 3 days at −20°C. The polymers were subsequently placed in a desiccator at room temperature for 5 days. From this procedure, dry 0.02% tPA/20% BSA/EVAc polymer rods were obtained, with an average weight of 40 mg, length of 12 mm, and diameter of 1.5 mm. The rods were cut into 3-mm discs, each weighing 10 mg.

Animal Population

New Zealand white rabbits (*Oryctolagus cuniculus*; Robinson Co., Winston-Salem, NC) weighing 1.5 to 2.5 kg were used. They were kept in standard animal facilities, one animal per cage, and given free access to Baltimore City water and rodent chow. The Johns Hopkins University Institutional Animal Care and Use Committee approved all experimental protocols.

Anesthesia Treatment

For the in vitro thrombolysis study, blood drawn from the central ear artery of a rabbit was used. Animals were anesthetized with a 2-ml intramuscular injection of a 5:1 mixture of ketamine (100 mg/ml; Abbott Laboratories, North Chicago, IL) and xylazine (100 mg/ml; Phoenix Pharmaceutical, Inc., St. Joseph, MO). For surgery, the animals were anesthetized with a 0.6-ml/kg intramuscular injection of a 5:1 mixture of ketamine (25 mg/ml) and xylazine (2.5 mg/ml). A 20-mg/kg intramuscular injection of ceftriaxone was administered prophylactically to all animals.

In Vitro Pharmacokinetic Study

The rate of release of tPA from 0.02% tPA-loaded EVAc polymers was determined in vitro using a tPA diagnostic kit (TintElize, Biopool International, Umeå, Sweden). Three tPA-loaded EVAc polymer disks weighing 10 mg each were suspended in separate screw-cap test tubes, each containing 1 ml of PBS-EDTA-Tween 20 buffer (pH 7.3), and incubated at 37°C. The polymers were then transferred to fresh 1 ml aliquots of PBS-EDTA-Tween 20 buffer at 1, 3, 6, 9, 12, 24, 48, and 72 hours. A 96-well enzyme-linked immunosorbent assay plate, precoated with goat anti-tPA immunoglobulin and loaded with nonimmune goat immunoglobulin and an indicator dye, was reconstituted using 50 μl of PBS-EDTA-Tween 20 buffer per well. To separate wells, 20-μl aliquots of each test sample were added. Twenty-microliter aliquots of four tPA standards (0, 10, 20, and 30 ng/ml) were also added to separate wells, followed by a 1-hour incubation on an orbital shaker (Bellco Glass, Inc., Vineland, NJ). To each well, 50 μl of conjugate (human anti-tPA Fab fragments labeled with horseradish peroxidase and suspended in 7 ml of PBS-EDTA-Tween 20 buffer) was added and allowed to incubate with agitation for 15 minutes. The contents of the wells were then discarded and each well was washed four times using PBS-EDTA-Tween 20 buffer. A substrate solution was made by mixing 300 μl of substrate concentrate (1,2 phenylenediamine dihydrochloride in buffer salts dissolved in 2 ml of distilled water) with 1.5 ml of distilled water and 300 μl H_2O_2 per 16-well strip. To each well, 100 μl of this substrate solution was added, followed by a 15-minute incubation. The enzymatic reaction was stopped using 100 μl of 1.6 M H_2SO_4 per well with an additional 5 minutes of incubation, yielding a color change from blue to yellow in the sample wells. The absorbance of each well was then read at 492 nm using an enzyme-linked immunosorbent assay plate reader (Beckman Coulter AD 340; Beckman Coulter, Inc., Fullerton, CA). The amount of tPA released at each time point was determined by comparison to a standard absorbency curve constructed using the 0, 10, 20, and 30 ng/ml tPA standards.

In Vitro Thrombolysis Study

Arterial blood was drawn from the central ear artery of the rabbits with a 25-gauge butterfly needle, and 6-ml aliquots of rabbit blood were transferred to 12 previously modified cell culture dishes in order to uniformly coat the bottom of each dish. The blood was then allowed to clot for 30 minutes at 37°C in a CO_2 incubator. Using forceps, a polymer was inserted onto the needle in the middle of each dish so that three dishes contained tPA polymers, three contained BSA polymers, three contained tPA/BSA polymers, and three contained blank polymers, and then the dishes were returned to the incubator. At 24 hours the cross-sectional area of thrombolysis was measured using a computerized image analysis system (MCID; Imaging Research, Inc., St. Catharines, Ontario, Canada), and the polymers were transferred to new Petri dishes filled with blood in the same fashion. Measurement and transferring of the polymers were repeated at 48 and 72 hours.

Areas of thrombolysis are expressed in square millimeters as the means ± the SEMs. These values were obtained by averaging the areas of thrombolysis obtained in each group. The Student t-test was used to compare the mean areas of the groups. Probability values less than 0.05 were considered significant.

In Vivo Studies

Surgery. Rabbits were anesthetized as described earlier. To minimize postoperative pain, a 0.04-mg/kg intramuscular injection of buprenorphine was given. Rabbits were then placed in a stereotactic

frame. Using standard sterile techniques, a midsagittal incision was made at a shaved area overlying bregma. The scalp was retracted with self-retainers, and the bregma was identified. Using the stereotactic frame, a location 4 mm anterior and 5 mm lateral (to the right) of bregma was measured, and a 0.5×0.5–cm bur hole was made. Once the dura was visualized, meticulous hemostasis was achieved using bone wax at the bur hole site.

To prepare the catheter system for blood infusion, we used a high-speed drill to file down the needle of a 20-gauge angiocatheter so that the sharp end of the needle was blunt. The length of this angiocatheter was measured, and a matching length of the stylette from an 18-gauge spinal needle was cut. This 18-gauge spinal needle stylette would serve as the stylette for the 20-gauge angiocatheter, to prevent any substances from entering the catheter when it was passed into the brain.

The 20-gauge angiocatheter with the blunt-tipped needle and 18-gauge stylette was fixed onto the stereotactic arm of the apparatus. Again, the entry point was localized at 4 mm anterior and 5 mm lateral (right) of the bregma. Then, from the dura, the catheter apparatus was inserted 5 mm deep, with the tip placed at the right frontal lobe on the lateral border of the internal capsule. The 20-gauge needle and 18-gauge stylette were then removed, leaving the plastic angiocatheter in its place. After verifying that no iatrogenic hemorrhage had occurred, we proceeded.

The 18-gauge stylette was then removed from the 20-gauge needle, and the needle was attached to a 1-ml tuberculin syringe previously filled with 0.5 ml of autologous arterial blood (drawn from the ear artery and allowed to clot for 20 minutes). The 20-gauge needle was primed with this clotting autologous blood, so that no air would be introduced into the brain during injection. The 1-ml tuberculin syringe with the attached 20-gauge needle was then reinserted into the plastic angiocatheter. This, again, placed the blunt-tipped needle at 4 mm anterior and 5 mm lateral of bregma and 5 mm deep. Then, we injected 0.2 ml of blood into the brain parenchyma and waited for 20 minutes for final clotting in situ. After that, an additional 0.1 ml of blood was injected as we withdrew the entire system from the brain, ensuring that the cavity caused by the angiocatheter system was also filled with blood and not air. Polymers were then implanted at the end of the surgery for both the sham and hemorrhage group. Polymer discs were implanted directly on the pial surface at the site of the bur hole. Then, bone wax was used to fill the osseous defect of the bur hole. The scalp was sutured using 3-0 nylon in a single running stitch.

For the toxicity study, only the polymer implantation was performed. No blood was injected.

Animal Protocol. Three days after blood placement, rabbits were anesthetized with a 0.6-ml/kg intramuscular injection of a 5:1 mixture of ketamine (25 mg/ml) and xylazine (2.5 mg/ml). Then, 2 ml of pentobarbital was administered by intracardiac injection. A midline sternotomy was performed, and the right ventricle was perforated. The left ventricle was cannulated and perfused with lactated Ringer solution at a rate of 100 ml/minute for a total of 10 minutes. Then, the animal was decapitated, and the brain was removed and placed in 5% paraformaldehyde for 7 days.

For the toxicity study, animals were killed at 3 months, and samples of brain, spinal cord, thyroid, esophagus, lungs, heart, stomach, pancreas, intestines, bladder, kidneys, liver, muscles, and blood vessels were fixed and sent for gross and histological pathological examination.

Brain Histology. After the brain had been fixed in 5% paraformaldehyde for 7 days, it was removed. The right frontal lobe was cut and placed in 2-methylbutane cooled with dry ice to $-30^\circ C$. This was then embedded in a mold using optimal cutting temperature compound and left in a freezer ($-40^\circ C$) until slicing. Then, the brains were cut into 90-μm-thick slices, which were placed onto microscope slides for subsequent measurement.

Thrombus Measurement. The National Institutes of Health Image program was downloaded from the Internet (http://rsb.info.nih.gov/nih-image/) and used for digitization of the brain slices. The intraparenchymal thrombus was measured from the brain sections, and the largest cross-sectional blood thrombus slice was used to calculate the volume by means of the following formula: volume = xyz/2.

Results

In Vitro Pharmacokinetics

In the in vitro study of release kinetics, the three tPA polymers (10 mg each, 0.02% tPA by weight) released a mean of 131 ± 13 ng, 7.6 ± 2.5 ng, and 7.8 ± 2.5 ng of tPA during each 24-hour period, measured at 24, 48, and 72 hours, respectively. These amounts correspond to a cumulative percentage release of 7.3% over 72 hours (Fig. 1).

In Vitro Thrombolysis

The mean area of thrombolysis per 24-hour interval for the three polymers in the tPA group was 302 ± 53 mm^2, 216 ± 80 mm^2, and 48 ± 29 mm^2 at 24, 48, and 72 hours, respectively (Fig. 2 *upper*). At these time points, the three polymers in the tPA/BSA group demonstrated an average area of thrombolysis of 358 ± 59 mm^2, 211 ± 51 mm^2, and 81 ± 45 mm^2, respectively. There was no significant difference between the average area of thrombolysis caused by tPA and tPA/BSA polymers. The three blank polymers and the three BSA polymers produced no thrombolysis ($p < 0.00001$). Figure 2 *lower* shows these data in terms of cumulative lysis over 72 hours.

In Vivo Toxicity

At 3 months after implantation of blank polymers in three animals and tPA polymers in three animals, all major organ tissues were examined grossly and histologically. There was no toxicity associated with tPA implantation.

In Vivo Thrombolysis

Whereas the six animals treated with tPA polymers had a mean thrombus volume of 1.43 ± 0.29 mm^3 (Fig. 3), the six animals treated with blank polymers containing BSA only had a thrombus volume of 19.99 ± 3.74 mm^3. The difference was statistically significant ($p < 0.001$, Student t-test). Coronal sections through the ICH of the animals treated with blank polymers containing BSA only (Fig. 4 *upper*) and with tPA polymers (Fig. 4 *lower*) grossly show the effects of the thrombolysis. The three animals receiving sham surgery had a mean thrombus measurement of 0.32 ± 0.27 mm^3.

Discussion

Intracerebral hemorrhage causes the highest morbidity and mortality of any form of stroke,[5,9,16] affecting 37,000 to 52,400 people each year in the US.[4,6,9,12,16] The 30-day mortality rate is 35 to 52%, and most survivors are left severely disabled.[6,10] Only 20% are independent at 6 months.[4] Most cases of ICH are attributed to systemic hypertension, degeneration and rupture of small arterioles, and advanced age; the most common sites of hemorrhage are the striatum, cerebellum, thalamus, and pons.[17] The damage from ICH is mainly due to the acute mass effect, focal damage to adjacent tissues, and poorly understood subacute mechanisms that continue to devastate the surrounding viable brain tissues.

No effective therapies for ICH currently exist, and treatment is primarily supportive. In contrast to the treatment of ischemic or subarachnoid hemorrhage, the acute manage-

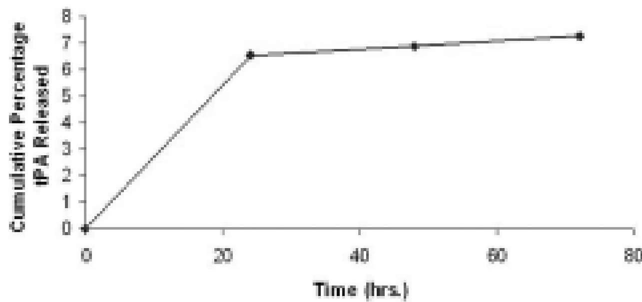

FIG. 1. Graph showing the mean cumulative percentage of tPA released by three tPA-loaded EVAc polymers over 72 hours in vitro.

ment of ICH has had only limited advances. Only four randomized medical trials[1,14,24,27] and six randomized surgical trials[2,3,8,11,13,28] have been published, and there is no conclusive evidence indicating an effective strategy for the treatment of ICH. The four medical trials compared the effect of steroids versus placebo,[14,24] hemodilution versus best medical therapy,[1] and glycerol versus placebo.[27] None of the authors of these four studies have demonstrated a statistical significance between the treatment and control groups. Furthermore, patients receiving steroids were more prone to infections, compared with their cohorts who received placebo.[14] The six randomized surgical trials also showed no conclusive evidence supporting surgical treatment. Other clinical studies have focused on thrombolysis of the ICH using a stereotactically implanted catheter guided to the site of hemorrhage.[12,23] In these studies, urokinase (5,000–10,000 IU) was given as a bolus at a set interval (every 6–8 hours) followed by aspiration of the liquefied thrombus, and it was shown to be effective in reducing the thrombus size by up to 57%.[12] However, there was a need for active intervention and manipulation of the catheter throughout the treatment.

Local controlled-release tPA polymers[19,20] can deliver thrombolytics to an area of hemorrhage, and use of the tPA polymers results in significant thrombolysis in a rabbit model of ICH. The local delivery of drugs to a specific site has several theoretical advantages, primarily because the blood–brain barrier prevents the entry of most drugs into the CNS. We have demonstrated that local or regional drug delivery using controlled-release polymers is a preferred strategy when treating certain CNS conditions such as tumors and edema.[19,21,22] We have previously shown in a rabbit model that controlled-release polymers can deliver sustained high local concentrations of drugs.[7,15] At 3 days, up to 50% of the brain parenchymal sections,[7] as well as the entire subarachnoid space,[15] were exposed to the drug. The drug concentration was sustained (up to 25%) even at Day 3.[7] In contrast, drugs that were directly injected into the brain demonstrated rapid disappearance within 3 hours.[7] In this study, we also found a direct correlation of the in vitro hemolysis area with the cross-sectional area of hemolysis in vivo. The main advantage of this technique is that implantation of a controlled-release polymer obviates the need to actively instill boluses of thrombolytics at regular intervals during the treatment period, thus theoretically reducing error, risk of infection, and time involved in the treatment.

In this study we made tPA polymers that released a total of 146 ng of tPA over the course of 3 days, with most of the

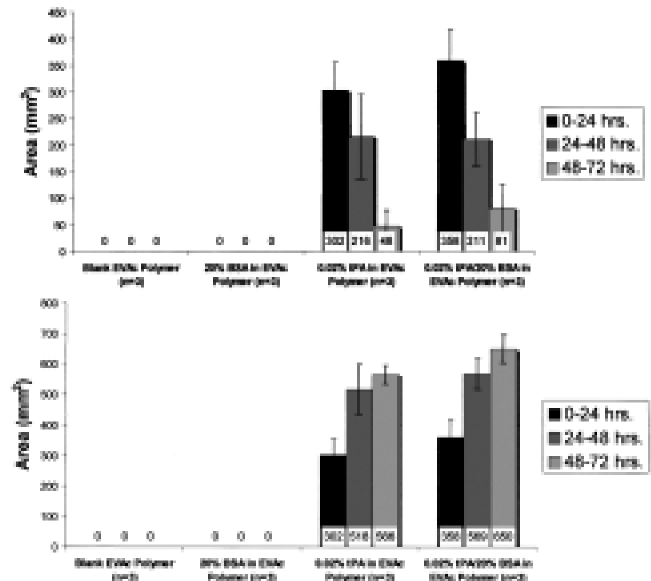

FIG. 2. *Upper:* Bar graph depicting mean area of thrombolysis associated with four types of EVAc polymers in vitro over 72 hours. *Lower:* Bar graph depicting mean cumulative area of thrombolysis associated with four types of EVAc polymers in vitro over 72 hours.

tPA released during the first 24 hours. By 72 hours the rate of tPA release was markedly decreased. This pattern correlated with the finding in the in vitro study, in which most of the thrombolysis occurred within the first 24 hours, with continuing thrombolysis through 72 hours. By 72 hours, the thrombolytic activity had markedly decreased. The results of the in vitro studies suggest that tPA activity occurs during the first 72 hours. In our rabbit model of ICH, animals that received blank polymers (containing only BSA) were compared with animals that were treated with tPA polymers at 72 hours, matching the maximum tPA activity time course. Whereas animals treated with tPA polymers had a statistically significant thrombus reduction to 1.43 ± 0.29 mm^3, animals treated with blank polymers had a thrombus volume of 19.99 ± 3.74 mm^3 ($p < 0.001$).

Therefore, tPA-loaded controlled-release polymers may be used in the acute treatment of ICH in which a single ste-

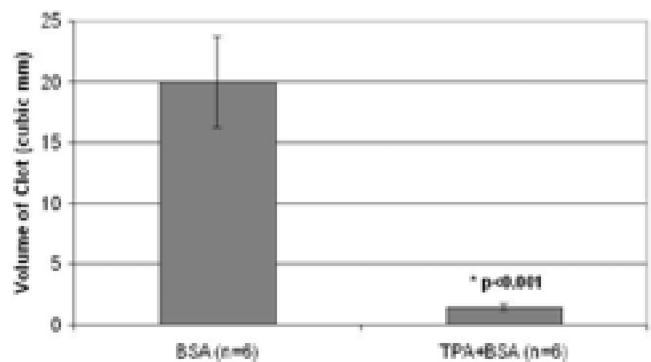

FIG. 3. Bar graph showing mean volume of thrombus at 72 hours in animals receiving BSA polymer and in animals receiving tPA/BSA polymer.

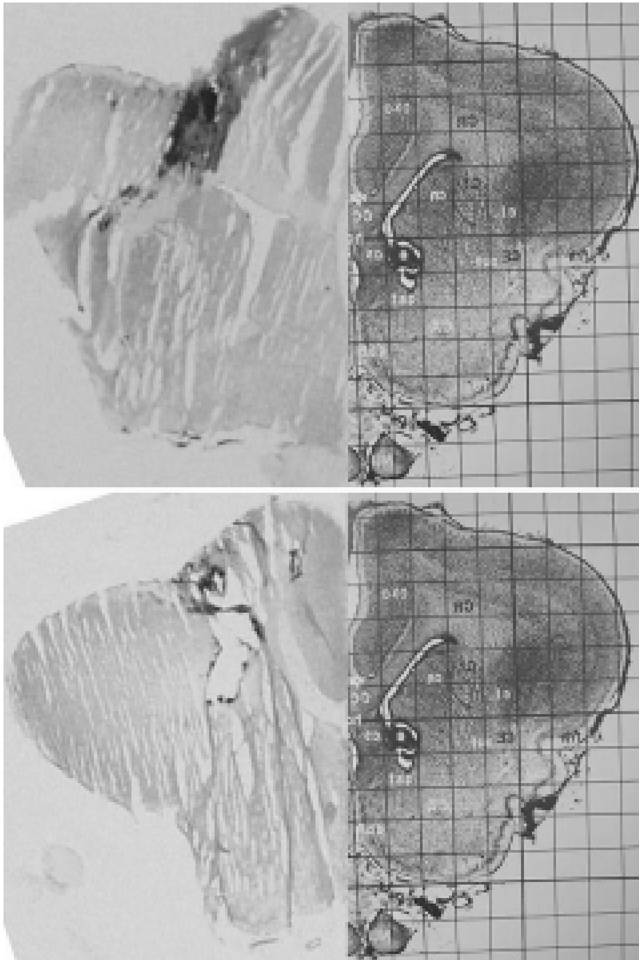

FIG. 4. *Upper:* Coronal section through the ICH showing intact thrombus in an animal treated with blank polymer containing BSA only. *Lower:* Coronal section showing a lysed thrombus cavity in an animal with ICH treated with tPA polymer. Original magnifications × 3.

reotactic procedure is required to implant the polymer at the site of hemorrhage.

Conclusions

Although the results of this study showed efficacy with a small clot size in the rabbit model, the clot size relative to the volume of the rabbit brain was of moderate size. For clinical application, where a much larger clot would be treated, a proportionately higher concentration of tPA would be needed but, in theory, would be expected to have similar pharmacokinetics and release kinetics over a proportionately larger volume. However, further studies are needed. Subacute processes associated with ICH may also be studied and ideally treated with controlled-release polymers, because polymers can be designed to release a wide range of drugs with variable pharmacokinetics.

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Manuscript received March 31, 2005.

Accepted in final form March 2, 2006.

This work was supported by a gift from Mr. and Mrs. J. Dorsey Brown to Dr. Tamargo.

Address reprint requests to: Rafael J. Tamargo, M.D., Division of Cerebrovascular Surgery, Department of Neurosurgery, The Johns Hopkins Hospital, 600 North Wolfe Street, Meyer 8-181, Baltimore, Maryland 21287. email: rtamarg@jhmi.edu.