Intracranial Clot Lysis With Intravenous Microbubbles and Transcranial Ultrasound in Swine

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Background and Purpose—Destruction of microbubbles by transcutaneous low-frequency ultrasound (LFUS) has been used to lyse adjacent clot and recanalize acutely thrombosed vessels. LFUS with intraarterial microbubbles has been shown to lyse cerebral clot rapidly in pigs without thrombolytic drugs. We hypothesized that intravenous platelet-targeted microbubbles with LFUS may be a rapid noninvasive technique to recanalize thrombosed intracerebral vessels.

Methods—After angiography, 0.5 cc of autogenous thrombus was injected into 1 ascending pharyngeal artery of a pig, occluding it and the rete mirabile. These vessels connect the carotid to the internal carotid and are the main cerebral blood supply. Saline control or intravenous decafluorobutane-sonicated dextrose albumin microbubbles tagged with a subtherapeutic quantity of glycoprotein 2b/3a receptor inhibitor eptifibatide (75 U/kg plus 12 cc of microbubbles administered over 21 minutes), or eptifibatide control, was given with transcutaneous temporal LFUS (1 MHz at 2.0 W/cm²) for 24 minutes. Angiography followed with scoring of declotting and flow. The same protocol was repeated on the contralateral side with the other test fluid so each animal received a saline control and either tagged microbubble or eptifibatide alone.

Results—Fifteen pigs completed the protocol with a mean clot age of 4.6 hours. Using tagged microbubbles, 6 of 8 achieved success compared with 0 of 7 receiving eptifibatide alone (P=0.007) and 1 of 15 receiving saline alone (P=0.02).

Conclusions—Intravenous platelet-targeted microbubbles combined with transcranial LFUS can rapidly open acute intracranial thrombotic occlusions. Further development for ischemic stroke therapy is justified. (Stroke. 2004;35:2407-2411.)

Key Words: stroke ■ thrombolysis ■ ultrasonics

Current therapy for acute ischemic stroke, 1 hour of intravenous tissue plasminogen activator (tPA) administered in the first 3 hours after onset, or intraarterial mechanical interventions or thrombolytics administered in the first 6 hours do not successfully reach many patients. Time constraints severely limit application of even the intravenous therapy, and angiography demands more time, very high skill levels, and sophisticated equipment. These therapies are also associated with increased hemorrhagic events within the brain. Effective, rapid, safe, and simple treatment is greatly needed.

Phantom, animal, and human studies have shown that low-frequency ultrasound (LFUS) increases tPA effectiveness within clots from 20% to 90%, probably from improved drug penetration into clot. Early human studies using this concept have been promising. Preliminary data from a new multicenter trial of the simple addition of continuous transcranial Doppler (TCD) to standard intravenous tPA therapy in human ischemic stroke patients have also shown marked improvement in the primary end points, confirmed the existence of a positive biological effect, and shown that 2 hours of continuous 2-MHz TCD ultrasound is safe in these patients.

Transcutaneous LFUS in combination with microbubbles also can rapidly dissolve thrombi without lytic agents. Multiple animal studies, including intracranial clot lysis in pigs, have confirmed this concept. Cavitation has been theorized to cause mechanical stress on the surface of the thrombus leading to its destabilization. Microbubble administration dramatically lowers the threshold for cavitation and increases the lytic activity of ultrasound. Because the bubbles are destroyed in the process, they must be constantly replaced for complete clot dissolution.

However, delivery of microbubbles to the clot has been problematic, with repeated intraclot injections and arterial
injections being most efficacious.10 Recent studies have demonstrated that microbubbles can be concentrated at the surface of thrombus by attaching a glycoprotein 2b/3a receptor antagonist to the bubbles, which increases their adherence to acute thrombus.14 Synergistic therapeutic effects of glycoprotein 2b/3a receptor antagonists have also been reported.15 In this study, we hypothesize that transtemporal LFUS used with microbubbles tagged with a low-dose glycoprotein 2b/3a receptor antagonist can recanalize acutely thrombosed intracranial vessels.

Materials and Methods

Microbubble Formulation and Stoichiometry
Decafluorobutane-sonicated dextrose albumin microbubbles (perfluorocarbon-exposed sonicated dextrose albumin [PESDA]) was formulated as described previously.16 Briefly, a 3:1 mixture of 5% dextrose and 5% albumin was hand-agitated with 8 mL of decafluorobutane gas, then sonicated for 80 seconds using an electromechanical sonicator. A total of 12 cc of PESDA was mixed with 75 U/kg eptifibatide (Integrilin; Key Pharmaceuticals; E-PESDA) to a total volume of 15 cc for 30-kg pigs.

The heat of interaction and stoichiometry epifibatide binding with PESDA was measured using isotherm titration calorimetry.17 This thermodynamic technique monitors for any chemical reaction generated by addition of incremental concentrations of epifibatide with PESDA microbubbles or a control solution consisting of the same quantities of dextrose and albumin without microbubbles. Measurements of heat produced or consumed by the interaction were performed with an Omega titration calorimeter (Microcal, Inc). From these values, the number of epifibatide molecules that bind to each microbubble was computed.

Study Protocol
Pigs (Oak Hill, Ewing, Ill) weighing 30 to 35 kg were studied with approval of the institutional animal care and use committee. Each was intubated and received general anesthesia with 3.5% isoflurane. Each received 40 mg of methylprednisolone (Solu-Medrol; Pharmacia and Upjohn Co) and 60 mg of ketorolac tromethamine (Toradol; Abbott Labs) to prevent pulmonary hypertensive allergic responses to human albumin and microbubbles, which can affect pigs.16 Selective ascending pharyngeal artery (APA) angiography was performed through a femoral approach. Autogenous venous clot, 0.5 cc, ranging from 2 to 7 hours in age, was injected into the APA and rete through the catheter. Angiography confirmed thrombosis and was repeated later to grade lysis.

Pigs were randomized to first receive either intravenous E-PESDA, 1.9 cc for 3 minutes up to 15 cc, or an identical epifibatide dose without microbubbles, or a control of the equivalent volume of saline intravenously, each fluid including 750 U of heparin. Each pig received a test fluid and saline control fluid treatments, 1 on each side. Transcutaneous LFUS (1 MHz at 2.0 W/cm²) was directed through the ipsilateral temporal bone for 24 minutes. A hand-held 10 cm² therapeutic transducer (Sonicat 716; Mettler Electronic) was set in pulsed mode (20% duty cycle), positioned behind the eye, and aimed slightly downward toward the base of the skull. At completion of the first treatment, the protocol was repeated on the contralateral side with the other fluid.

Scoring
Three observers scored angiographic images and reached consensus using a variation of the thrombolysis in myocardial infarction flow scale of 0 to 3 and a previously described declotting scale (0=no clearing; 1=<30% lumen clearing; 2=30% to 70%; 3=70% to 90%; and 4=>90% clearing).8–10 Success was defined as declotting of ≥3 and flow of ≥2 scored in the APA and rete individually at 24 minutes. The scores from the 2 vessels were totaled for statistical analysis (thus, optimal scores were 8 for declotting and 6 for flow).

Declotting and flow scores used Wilcoxon signed rank tests for paired observations and Wilcoxon rank sum test for unpaired observations. McNemar test was used for paired statistical analysis of overall success rate and Fisher exact test for unpaired comparisons. A P value of <0.05 was considered significant.

Results
Isotherm titration studies demonstrated an exothermic reaction between PESDA and epifibatide, with a computed heat of interaction of −34.9 kcal/mol of epifibatide. On the basis of the titration curve and known molecular weights, 6.7×10ⁱ³ epifibatide molecules bind to each microbubble.

Fifteen pigs completed the protocol. Clot age at the start was similar in all test groups (mean 4.6 hours). Angiographic success (Figure 1) was achieved in 6 of 8 with E-PESDA, 0 of 7 with epifibatide (unpaired P=0.007) and in 1 of 8 saline controls (paired P=0.03; Table). Mean total declotting scores (optimum 8) for E-PESDA were 6.3±1.8, for epifibatide

Figure 1. Selective angiograms of the ascending pharyngeal artery. (A) Embolic occlusion of the artery and rete after clot injection (arrows). (B) Complete recanalization after E-PESDA therapy. The rete mirabile are tangles of tiny arteries connecting the ascending pharyngeal artery to the internal carotid. Note filling of the opposite rete through the broad midline connection and normal filling of cerebral vessels.
4.0±2.6, unpaired versus E-PESDA (P=0.06), and saline controls were 3.6±1.7 (paired P=0.02). Mean total flow scores (optimum 6) were E-PESDA 3.3±1.5, eptifibatide 1.1±0.9 (P=0.008) versus E-PESDA unpaired, and saline 1.6±1.0 (P=0.02) versus E-PESDA paired.

Activated clotting times were not increased by treatments (mean 144 seconds before and 142 after). Pathological and histological study of 3 brains showed no sign of residual clot or microbubble within E-PESDA–treated vessels and no endothelial damage or hemorrhage. No sign of focal infarct was identified in either the treated or control side of the brains.

Discussion

Current therapy for acute ischemic stroke must be improved. Both intravenous thrombolysis and arteriographic approaches provide too few successes, have too many complications, and reach too few patients for a host of reasons.1,2 Alexandrov5 leads one of the most promising current clinical trials in human ischemic stroke therapy using standard tPA augmented with continuous TCD. Preliminary reports from the CLOTBUST Trial show positive trends in outcomes and safety equivalent to tPA alone in 2-hour trials (A.V. Alexandrov, unpublished data, 2004). It supports that continuous TCD enhances tPA-associated recanalization and proves existence of a biological effect of ultrasound-enhanced thrombolysis. Some early recanalization occurred with continuous TCD in 73% compared with 50% in controls, and the primary end points were achieved in 49% compared with 30% (P=0.02). Although the primary occlusive thrombi in human ischemic strokes may be formed long before they embolize and give symptoms in many cases, thrombolysis and thrombolysis augmented with TCD still produce improved outcomes. Whether this is attributable to lysis of acute secondary clot adjacent to the embolus (which may even be insoluble material), lysis of old and new clot in the primary thrombus, or a combination of actions, the effect remains positive. However, this technique requires very skilled ultrasonographers because the beam is only 3-mm wide. Very precise aiming and frequent adjustments are necessary to get these results. A broader beam technique suitable for use by less sophisticated operators has considerable appeal (Figure 2).

The mixture of a subtherapeutic dose of the glycoprotein 2b/3a receptor inhibitor eptifibatide and microbubbles appears to tag the microbubbles and allows successful and rapid clot dissolution with LFUS using only intravenous administration. The isotherm titration calorimetry does not identify what the binding mechanism is because we do not know the kinetics and structures of the complexes. However, the high exothermic heats indicate that eptifibatide is reacting with microbubbles to most likely form noncovalent bonds. In other words, at mixing, the eptifibatide molecules rapidly penetrate the surface of the microbubbles, generating a variety of van der Waals contacts between the drug and microbubbles.

Previously, intraarterial microbubbles with LFUS have performed well in this model but required angiography and

![Figure 2. A contrast-enhanced computerized tomographic axial view of the rete and surrounding structures shows the paired rete (arrows) and the 5- to 7-cm distances and various boney structures between the temporal skin surface where the transducers were placed (*) and the target rete. Note that the ascending pharyngeal artery is below the base of the skull, yet even in the shadow of this bone, adequate ultrasound reaches the target to provide lysis.](http://stroke.ahajournals.org/)

### Results and Statistics

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NA indicates paired comparison was equal and cannot be assigned a McNemar P value; E, eptifibatide; EP, PESDA tagged with eptifibatide (E-PESDA).
all of its problems and delays. Intravenous microbubble delivery is much simpler and requires no great skill or complex equipment. Transcranial ultrasound delivery with a 3-cm-diameter beam is also simple and does not require intraarterial cannulation or angiography. The large therapeutic ultrasound transducer system is inexpensive and does not require extreme precision in aiming the beam. Clot lysis and restored flow were achieved in only 24 minutes, a very promising schedule for emergency management of stroke.

The most devastating of current therapy complications are related to bleeding associated with thrombolytic drugs or reperfusion. Our technique achieved success without thrombolytic drugs and without increasing clotting times, so reduced risk of hemorrhage and improved outcomes are expected. Alternatively, combinations of very low doses of thrombolytics and microbubble therapy may be another fruitful avenue of research to avoid these hemorrhagic events.

The 0.5-cc clot volume used here corresponds to that in many clinical strokes, although these volumes vary widely, and smaller clots can certainly cause severe strokes. Intravenous microbubbles and LFUS have proven quite successful with small clots (<0.3 cc) lysis but has been less successful with large clot burdens. Platelet-targeted microbubbles may have wide application in small clot lysis and prove useful as a first emergency step leading to angiographic approaches on larger clots or as follow-up to those efforts. The smaller fragments that cannot be chased with mechanical arterial devices or the residual from these devices may respond to this approach. Small clots have favorable surface-to-volume ratios making this clot surface attack more likely to succeed.

Study Limitations
Extrapolating our data to a human setting has limitations. The actual age of the thrombus in patients with acute ischemic stroke is unknown and may be either fresh or quite old. Nonetheless, we did use thrombi that are old enough (2 to 7 hours) to approximate the time frame between the onset of clinical symptoms and initiation of therapy. This may closely imitate secondary clot in the arteries adjacent to the original embolic material. This new clot can contribute to the ischemic event in areas beyond the original insult and may be quite vulnerable to lysis.

Secondly, one cannot exclude that eptifibatide or heparin may have had some clot-dissolving effect independent of microbubbles or that temporal effects of the sequential design of E-PESDA and eptifibatide animals may have affected results. However, the cumulative dose of eptifibatide was well below the dose required to inhibit platelet aggregation in clinical trials, tests with eptifibatide did not show therapeutic effect, and these were very similar to saline controls. Also, identical subtherapeutic levels of heparin were included in the microbubble, the eptifibatide, and control saline fluids.

The specific animal model is a limitation, because the rete mirabile is not present in humans. This tangle of tiny arteries blocks direct access to the internal carotid artery so that clot cannot be directly injected into cerebral vessels and reliably cause stroke. Angiographic scoring here includes only the APA and rete on the affected side. Scoring of internal carotid circulation was not included because extensive collaterals and the rete protect these vessels from embolization so effectively. Flow changes and thrombosis there are almost never seen, and brain perfusion is not measured effectively.

Pathological examination of the 3 brains studied acutely here did not show evidence of stroke or ultrasound damage, but acute ischemic changes can take hours to appear pathologically. Ongoing survival studies with MRI and neurological assessment, as well as delayed pathological study, may show subtle findings at 24 hours that were not yet apparent from these immediate pathological exams. Other animal model considerations include the fact that the overall head size of a pig is similar to humans, but pig brains are much smaller, and surrounding soft tissues are larger. Nonetheless, the clot volume was similar to humans, swine physiology is similar, and similar physical dimensions suggest that temporal ultrasound delivery will be similar.

Finally, the toleration of ischemic brain to low-intensity ultrasound levels used here in combination with microbubbles has not yet been fully studied. However, animal experiments in normal rats without tPA or microbubbles show that levels ≤2.0 W/cm² produce no significant heating or harm to the brain, and the CLOTBUST Study showed no increase in significant intracranial hemorrhage with therapeutic tPA levels.

Future Direction
Extensive studies in animal models are required to survey several possible variations in this technique before moving to human trials. A better survival animal model such as rabbit or rat would help compare efficacy and safety of LFUS plus microbubbles, LFUS and thrombolytic drugs, and various combinations. Testing of combinations of very low-dose tPA and microbubbles seems particularly important because the mechanisms appear to be different and may well be additive. Very low-dose tPA may avoid some of the bleeding complications of standard tPA therapy. Other testing, including that of improved ultrasound delivery systems, may be better done in the pig model or human cadavers. Clinical demand for improved therapy has led to the human stroke trials using current TCD technology and the exciting CLOTBUST results, but a survey of the most likely improved techniques would be better served in further basic investigations and direct animal comparisons, while more human data are acquired with current techniques.

Summary
Intravenous administration of platelet-targeted microbubbles successfully augments the effect of transcutaneous LFUS in dissolving acute intracranial thrombi without thrombolytic drugs. With further refinement in bubble technology and ultrasound delivery, this concept may be the key to earlier and safer stroke therapy. It may become an effective and superior alternative to current fibrinolytic therapy in the acute treatment of stroke.

References
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