Effect of Ultrasound on Tissue-Type Plasminogen Activator–Induced Thrombolysis

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Background. The efficacy of fibrinolytic therapy is limited by the small surface area of the clot that is available for the binding of the thrombolytic agent, such as tissue-type plasminogen activator (t-PA). We hypothesized that exposure of the clot to ultrasound during thrombolytic treatment could enhance lysis through perturbation of the thrombus, which would expose additional fibrin binding sites for t-PA.

Methods and Results. Whole human blood clots containing radiolabeled fibrinogen were incubated in vitro for 200 minutes with Tris–albumin buffer containing t-PA at concentrations ranging from 3 to 3,000 IU/ml. In paired experiments, one of the clots also was exposed to intermittent ultrasound (1 MHz, 1.75 W/cm²) throughout the experiment. The ultrasound was delivered as a 2-second exposure followed by a 2-second rest interval. The overall difference in mean clot lysis between thrombi receiving ultrasound and those receiving no ultrasound was significant (p<0.001) at all concentrations of t-PA. For clots incubated with t-PA at a concentration of 300 IU/ml, ultrasound increased the percent lysis at 200 minutes from 42±5% (mean±SEM) to 64±10%. In six paired experiments in a rabbit jugular vein thrombosis model, rabbits received 1 mg t-PA alone or t-PA and intermittent ultrasound (1 MHz, 1.75 W/cm²) for 200 minutes. For rabbits receiving ultrasound and t-PA, lysis was 55±11% at 100 minutes compared with 30±12% for rabbits receiving only t-PA. Lysis was 6±10% for rabbits (n=4) receiving ultrasound alone. No evidence for tissue damage was noted in rabbits exposed to intermittent ultrasound.

Conclusions. Exposure of whole blood clots in vitro to intermittent ultrasound combined with t-PA caused a significant enhancement of thrombolysis compared with t-PA alone. Intermittent ultrasound also showed a trend toward enhancement of t-PA-induced clot lysis in an animal thrombosis model. These data suggest that noninvasive intermittent ultrasound may be a useful adjunct to thrombolytic therapy.

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Key Words • fibrinolysis • ultrasound • thrombosis • plasminogen activators

Although tissue-type plasminogen activator (t-PA) is efficacious for lysis of coronary thrombi and pulmonary emboli, relatively high concentrations are required.1-3 This may result in part from the paucity of fibrin binding sites for t-PA on the surface of whole blood clots.4 In the absence of binding to fibrin, t-PA has greatly reduced efficacy in activating plasminogen and inducing clot lysis.5 In clinical practice, t-PA can be directly injected into the site of thrombus with percutaneous transluminal catheters rather than infused systemically.6,7 Presumably this approach improves lysis by increasing the surface area of fibrin available for t-PA binding.

Ultrasound has been used without exogenous t-PA to disrupt peripheral arterial and venous thrombi in animal models8-11 and to open atherosclerotic occlusions of peripheral arteries in selected patients.12,13 To achieve these effects, ultrasound was used at a frequency (20 kHz) and intensity that caused cavitation, thereby disrupting tissues with low elasticity, such as atheromas and thrombi.13,14 The ultrasound was delivered through catheters to prevent damage to normal tissues near the sites of the occlusions.

Kudo and coworkers15-17 are the first to report the use of noninvasive ultrasound to increase the efficacy of systemic t-PA. They found that transcutaneous ultrasound that was delivered in a continuous mode at a frequency of 200 kHz could enhance t-PA–induced fibrinolysis in a canine model of femoral arterial thrombosis.15-17 Ultrasound that is used in physiotherapy is delivered at a frequency of 1 MHz, which can cause acoustic streaming, or wave–medium interactions that promote agitation of solute and microparticulate matter without inducing cavitation or tissue damage.18 This property of ultrasound suggested that it might have the potential for gentle perturbation of a clot, which would expose additional fibrin for t-PA binding. The ability of noninvasive ultrasound, delivered at a frequency of 1 MHz in an intermittent mode, to enhance t-PA activity was therefore studied in vitro and in vivo using the rabbit jugular vein thrombosis model.19

Methods

Materials

Single-chain human recombinant t-PA (lot number N9102, specific activity 580,000 IU/mg) was purchased...
from Genentech, Inc., South San Francisco, Calif.; thrombin (2,200 National Institutes of Health [NIH] units/mg) was from Sigma Chemical Co., St. Louis, Mo. Lysine Sepharose and Sephadex G-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden; iodogen was from Pierce Chemical, Rockford, Ill.; and $^{125}$I was purchased from Amersham Corp., Arlington Heights, Ill. Human serum albumin was from Miles Inc., Elkhart, Ind. Plasminogen was purified from human fresh frozen plasma by affinity chromatography on a lysine-Sepharose column. Plasminogen was in the Glu form as assessed by sodium dodecyl sulfate gel electrophoresis and had a specific activity of 20 Committee on Thrombolytic Agents (CTA) units/mg.

Preparation of $^{125}$I-Labeled Fibrinogen

Fibrinogen was purified from fresh human plasma by glycine precipitation and was radiolabeled with $^{125}$I by the iodogen technique. Two milliliters of fibrinogen (1.8 mg/ml) in 0.05 M Tris–HCl, 0.10 M NaCl, 0.025 M sodium citrate, pH 6.8 was mixed with 400 $\mu$Ci $^{125}$I at 22°C for 2–3 minutes in a scintillation vial precoated with 20 $\mu$g iodogen. Unbound $^{125}$I was separated from labeled fibrinogen with a Sephadex G-25 column. The radiolabeled fibrinogen was 88% clottable and had a specific activity of $3.0 \times 10^7$ cpm/mg.

Ultrasound

A 1-MHz ultrasound generator (Amrex Synchrosonic U50, Amrex-Zetron, Inc., Hawthorne, Calif.) was used to provide continuous-mode or intermittent-mode ultrasound output. For intermittent ultrasound, the “on” interval was 2 seconds, followed by a rest interval of 2 seconds. Ultrasound was tested at two different intensities (expressed as spatial-average temporal-average intensities) of 0.375 W/cm$^2$ and 1.75 W/cm$^2$. Ultrasound was delivered through water from a planar transducer (surface area, 23 cm$^2$), which was 6 cm from the thrombus for both in vitro and in vivo experiments. At this distance, ultrasound was delivered as a cylindrical beam. The attenuation was less than 1% in a 6-cm path in water. In tissue, the intensity was reduced by 11% in a 1-cm path.

In Vitro Thrombolysis

Blood was obtained by venipuncture from one normal volunteer under a protocol approved by the Human Use Committee of the Walter Reed Army Institute of Research. Whole blood clots were prepared by adding thrombin (50 $\mu$L, 15 NIH units) to a mixture of 3 ml freshly obtained blood and 300 $\mu$L $^{125}$I-labeled fibrinogen (0.7 $\mu$Ci) in a 10×75 mm parafilm tube.

After incubation at 22°C for 2 hours, the clots were added to two beakers that were in a water bath maintained at 37°C (Figure 1). The beakers each contained 100 ml supernatant. In one of the two beakers, the ultrasound transducer was placed in direct contact with the supernatant, and the clot was at the bottom of the conical beaker, or 6 cm from the face of the transducer. The surface area of each beaker was 43 cm$^2$. The walls of the Pyrex beakers were 2 mm thick, and the angle between each beaker and the external perpendicular support was 23°C. Conical beakers were used so that the thrombus would not drift out of the range of the ultrasound beam. The temperature of the supernatant was monitored throughout the experiment with a thermometer that was accurate to 0.1°C. Each paired experiment (at a given t-PA concentration and buffer composition) consisted of two clots, one of which was exposed to ultrasound. The supernatant was either 100 ml Tris–albumin buffer (0.15 M NaCl, 0.02 M Tris–HCl, pH 7.40 containing 1% human serum albumin) or 100 ml human plasma obtained as fresh frozen plasma. In the six paired experiments using fresh frozen plasma, six units of plasma, each from a different donor, were used; plasma from a single unit was used for each paired experiment. After addition of the clot to the supernatant, fibrinolysis was initiated by addition of t-PA at final concentrations ranging from 3 to 3,000 IU/ml.

Fibrinolysis was monitored by determining the radioactivity of the whole clot in a gamma counter (Searle model 1185, Searle Diagnostics, Inc., Des Plaines, Ill.) before addition to the supernatant. Immediately after addition of the clot to the beaker and before addition of the t-PA, a 200-$\mu$L aliquot of the supernatant was tested for radioactivity, followed by serial sampling throughout the 200-minute experiment. Sampling intervals ranged from 10–15 minutes during the initial phase of the lysis to 30–40 minutes after the plateau was achieved. The residual radioactivity in the clot was determined at 200 minutes.

Percent lysis was calculated according to the following formula:

$$\frac{500(\text{supernatant cpm time } x - \text{supernatant cpm time 0})}{\text{thrombus cpm time 0} - \text{background}} \times 100$$

In Vivo Thrombolysis

The experimental protocol, which was approved by the Walter Reed Army Institute of Research Animal Committee, used male New Zealand White rabbits weighing between 3.0 and 3.5 kg.

Thrombosis model. The rabbit jugular vein thrombosis model was chosen because of its established value in the testing of t-PA for clinical use and the relative simplicity of the required surgical procedure and animal care. Anesthesia was induced with the intramuscular injec-
A vein.19,23

**Figure 2.** Schematic of equipment used in the rabbit experiments for the administration of tissue-type plasminogen activator, delivery of ultrasound, and monitoring of soft-tissue temperature. The neck of the rabbit was in contact with the water bath. The ultrasound transducer was coated with transducing gel and placed against a silastic membrane at the bottom of the water bath.

Fibrinogen mixed then into the vein. 0.038-in.-o.d. tubing, 2.5% halothane (A.M. Bickford, Wales, N.J.) administered through 0.1 mg/kg xylazine (Rompun, Miles Laboratories, Inc., Shawnee, Kan.). The animals were intubated, and anesthesia was maintained with 1.5–2.5% halothane (Halocarbon Laboratory, Inc., Hackensack, N.J.) administered through a model 50122 Bickford vaporizer (A.M. Bickford, Inc., Wales Center, N.Y.).

The jugular vein was isolated as previously described with ligation of all side branches except the facial vein.19,23 A venotomy in the facial vein then allowed a 0.038-in.-o.d. polyethylene catheter (Intramedic tubing, Clay Adams, Parsippany, N.J.) to be introduced and a 1-ml aliquot of blood to be withdrawn. The blood was then mixed with 100 μl (1 μCi) 125I-labeled human fibrinogen and kept on ice to prevent clotting. Next, the jugular vein was atraumatically occluded proximal and distal to the facial vein with microvascular clamps (Scanlan International, Inc., St. Paul, Minn.). A 4-0 silk thread soaked in human thrombin solution was placed into a small polyethylene catheter, which was inserted into the venotomy in the facial vein and guided into the jugular vein. The radiolabeled blood (0.5 ml) was introduced into the jugular vein through the facial vein to create a thrombus around the 4-0 silk thread. The catheter in the facial vein was removed, the facial vein was ligated around the thread, and the end of the thread was brought out to the skin through a small puncture to facilitate percutaneous removal at the completion of the experiment. The microvascular clamps were removed, and the skin was closed.

A temperature probe (2400 series temperature probe, Electromedics, Inc., Englewood, Colo.) to be used for temperature monitoring during the experiment was inserted into the neck through a separate skin puncture. The animal to receive ultrasound was positioned on a specially designed treatment table with an enclosed water bath maintained at 36.5–38.5°C (Figure 2). The other animal was positioned in a similar fashion on a circulating water warming blanket also maintained at 36.5–38.5°C.

**Experimental protocol.** To determine the effect of ultrasound on t-PA-induced fibrinolysis, a series of paired experiments were performed in which both animals received t-PA and one of each pair received ultrasound in addition to the t-PA. Intermittent ultrasound (1 MHz, 1.75 W/cm², with a 2-second on interval and a 2-second rest interval) was delivered throughout the experiment to the neck of the animal from a fixed transducer through a water bath 6 cm deep (Figure 2). The distance was chosen so that the ultrasound beam could provide adequate coverage of the site of potential thrombosis in a human model. Controls for the rabbits receiving t-PA and ultrasound were four rabbits that received ultrasound and no t-PA.

In six paired experiments, animals received t-PA at a dose of 1 mg delivered intravenously during the first 30 minutes of the experiment. An additional six pairs of animals received t-PA at a dose of 2 mg. In this group, 1 mg t-PA was administered during the first 30 minutes, followed by 1 mg from 35 to 185 minutes. One control animal in this group died of postoperative respiratory insufficiency; therefore, only five paired studies are reported.

All animals received maintenance fluid and t-PA through a marginal ear vein cannula ipsilateral to the jugular vein thrombus. One milligram of t-PA was diluted in 5 ml of 0.3 M NaCl, 0.01% Tween 80 (Aldrich Chemical Co., Arlington Heights, Ill.) and administered with a constant-infusion pump (Sage Instruments, Cambridge, Mass.).

Fibrinolysis was monitored by obtaining a 2-minute count of radioactivity over the neck of each animal at time 0 (before the infusion of t-PA) with a calibrated gamma ratemeter (model 2221 scalar ratemeter, Lumen Measurements, Inc., Sweetwater, Tex.).23 This was followed by serial counts throughout the experiment, with background counts obtained as well at each time point.

Percent lysis was calculated according to the following formula:

\[
\frac{\text{cervical cpm time 0} - \text{cervical cpm time x}}{\text{cervical cpm time 0} - \text{background}} \times 100
\]

Plasma fibrinogen and serum plasminogen were measured with functional assays as previously described.24

**Toxicity Studies**

To determine the extent of tissue injury sustained from insonation, the levels of creatinine phosphokinase (CPK; Encore Chemistry System, Baker Instruments, Inc., Allentown, Pa.) were measured in serum at times 0, 200 minutes, 24 hours, and 7 days in the pairs of rabbits receiving 1 mg t-PA in the presence and absence of ultrasound.

A blinded histological examination for pulmonary emboli was performed in four rabbits treated only with t-PA and in eight rabbits that received combined t-PA and ultrasound.

The effect of insonation on jugular veins in the absence of thrombosis was studied histologically in three pairs of animals. Each pair underwent a sham operation to include catheterization of the jugular vein. Then one animal in each pair received 200 minutes of intermittent ultrasound (1 MHz, 1.75 W/cm²) directed
at the jugular vein. Seven days later, the animals were killed with a 5-ml intravenous injection of sodium pentobarbital (Euthanasia-6). The jugular vein segments and lungs were removed and immersion-fixed in neutral buffered 10% formalin. Tissue was subjected to blinded histological evaluation for inflammatory changes in vein segments.

Statistical Methods

Summary thrombolysis data are reported as mean ± SEM. ANOVA was used to determine the statistical significance of differences in clot lysis caused by effects of 1) ultrasound (alone or in combination with t-PA), 2) concentration of t-PA, and 3) time of measurement. This analysis included time as a repeated-measures factor and took into account the paired nature of the experimental design for comparing clots receiving ultrasound and t-PA with those receiving t-PA alone. This feature results because each experimental run at a given t-PA concentration included two clots studied in parallel: one receiving ultrasound and t-PA, the other t-PA alone (see Figure 1). The 50-minute and 200-minute time points (the repeated-measures factor) were analyzed to determine early and late effects of insonation (reflecting rate and extent of fibrinolysis) for all in vitro experiments. Similar analyses were used for the in vivo data at 50 and 100 minutes, a time when fibrinolysis had reached a plateau. The t test (paired or unpaired as appropriate) was used when only two groups were compared. Observed significance levels (probability values), involving effects of ultrasound derived from t tests or from one-degree-of-freedom F tests [F (1, df)], were two-sided. A value of \( p < 0.05 \) was considered to be significant. Analyses based on transformed data (logs) yielded similar results. The ordinary (Pearson’s) correlation coefficient was used as a measure of correlation between two variables.

Results

Comparison of Different Ultrasound Modes on t-PA–Induced Clot Lysis In Vitro

The optimal intensity of ultrasound for enhancement of clot lysis was determined by exposing blood clots in Tris–albumin buffer containing t-PA (300 IU/ml) to ultrasound at a frequency of 1 MHz and an intensity of 1.75 W/cm² or 0.375 W/cm² for 200 minutes. Intermittent or continuous ultrasound at an intensity of 0.375 W/cm² did not enhance thrombolysis. Continuous ultrasound at an intensity of 1.75 W/cm² increased the temperature of the medium from 37.0°C to 39.0°C within 10 minutes of initiation and maximally by 5°C during the course of the 200-minute experiment. Therefore, this mode was not evaluated further. However, intermittent ultrasound at an intensity of 1.75 W/cm² caused significant enhancement of lysis at 200 minutes (64±10% versus 42±5%, \( p < 0.05 \)) without increasing the temperature of the surrounding medium by more than 1°C. Therefore, intermittent ultrasound at an intensity of 1.75 W/cm² and a frequency of 1 MHz was chosen for all further studies.

Effect of Intermittent Ultrasound (1 MHz, 1.75 W/cm²) on Clot Lysis Induced With Different Doses of t-PA in Buffer

Experiments were conducted in the absence and presence of intermittent ultrasound and t-PA at four different concentrations (3, 30, 300, and 3,000 IU/ml) to determine whether ultrasound affected the rate and extent of t-PA–induced fibrinolysis (Figure 3). Differences in mean clot lysis caused by effects of ultrasound

![Graphs showing effect of intermittent ultrasound (1 MHz, 1.75 W/cm²) on whole blood clot lysis induced by different concentrations of tissue-type plasminogen activator (t-PA) in Tris–albumin buffer. The ultrasound was delivered during 2 seconds with a 2-second rest interval.](image-url)
and t-PA concentration were determined by repeated-measures ANOVA, with the 50-minute time point reflecting the rate of fibrinolysis and the 200-minute time point reflecting the extent of fibrinolysis. The overall differences in mean clot lysis among the four t-PA concentrations were significant \( F(3,10)=8.3; p<0.005 \), with the extent of fibrinolysis increasing in a dose-related manner for thrombi receiving ultrasound or no ultrasound.

The overall difference in mean clot lysis between thrombi receiving ultrasound and no ultrasound was highly significant \( F(1,10)=52.2; p<0.001 \), with differences in time course consistent across all four t-PA concentrations. There was also a significant difference in the two groups (ultrasound versus no ultrasound) with respect to the rate of lysis as measured at 50 minutes \( p<0.001 \) and extent of clot lysis as measured at 200 minutes \( p<0.001 \) when analyzed separately. At the lowest concentration of t-PA (3 IU/ml), ultrasound caused a 100% enhancement of lysis as determined at 200 minutes and an approximate 50% increase at the higher t-PA concentrations (Figure 3).

**Effect of Plasma and Plasminogen on Fibrinolysis Induced by t-PA and Ultrasound (1 MHz, 1.75 W/cm²)**

In paired experiments, clots were incubated with fresh frozen plasma to which t-PA had been added at a final concentration of 300 or 3,000 IU/ml, and one clot in each pair was exposed to intermittent ultrasound (Figure 4). Ultrasound induced a significant increase in the extent of thrombolysis as determined with the repeated-measures ANOVA using 50- and 200-minute data at both t-PA concentrations \( F(1,4)=36.9; p=0.004 \). In all six paired experiments (three pairs at 300 IU/ml t-PA and three pairs at 3,000 IU/ml t-PA), the thrombus exposed to ultrasound and t-PA underwent greater lysis than the thrombus incubated with t-PA alone.

When clots were exposed to ultrasound and t-PA at a concentration of 300 IU/ml, the lysis at 200 minutes was 75±7% compared with 50±2% when clots were incubated with t-PA alone. Furthermore, ultrasound reduced the time to reach 50% lysis from 200 to 96 minutes.

For the clots exposed to t-PA at a final concentration of 3,000 IU/ml, the mean time to reach 50% lysis was reduced from 98 to 48 minutes in the presence of ultrasound. Clot lysis at 200 minutes was 91±11% in the presence of sonation compared with 62±5% in the absence of ultrasound.

Fresh frozen plasma derived from a single donor was used in each paired experiment. The mean plasminogen concentration in the plasmas \( n=6 \) used for these experiments was 3.8±0.2 CTA units/ml. In additional experiments, clots were incubated with t-PA (3,000 IU/ml) in Tris-albumin buffer containing purified plasminogen at a concentration of 1.2 CTA units/ml. The same degree of lysis was achieved at the lower concentration of plasminogen (Table 1).

The results of thrombolysis experiments with 3,000 IU/ml t-PA in Tris-albumin, plasma, and Tris–albumin containing plasminogen are summarized in Table 1. In the absence of ultrasound, there was no significant

<p>| Table 1. Effect of Intermittent Ultrasound on Clot Lysis Induced by t-PA in Buffer, Plasma, and Buffer Containing Purified Plasminogen |
|------------------|------------------|------------------|
| % Lysis at 50 minutes | % Lysis at 200 minutes |</p>
<table>
<thead>
<tr>
<th>US</th>
<th>No US</th>
<th>p</th>
<th>US</th>
<th>No US</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA (3,000 IU/ml)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Buffer</td>
<td>45±12</td>
<td>33±7</td>
<td>0.07</td>
<td>73±12</td>
<td>62±15</td>
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<tr>
<td>Plasma</td>
<td>50±9</td>
<td>33±4</td>
<td>0.05</td>
<td>91±11</td>
<td>62±5</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>54±5</td>
<td>35±2</td>
<td>0.02</td>
<td>106±6</td>
<td>77±4</td>
</tr>
<tr>
<td>Control rabbits (no t-PA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Buffer</td>
<td>4±1</td>
<td>1±1</td>
<td>0.04</td>
<td>8±1</td>
<td>3±1</td>
</tr>
</tbody>
</table>

- t-PA, tissue-type plasminogen activator; US, ultrasound; US frequency and intensity, 1 MHz and 1.75 W/cm². Values of \( p \) are those for the given time points at the single dose of t-PA. The differences in mean clot lysis incubated in buffer, plasma, or buffer with plasminogen are not significant \( p>0.05 \). Three paired experiments (ultrasound vs. no ultrasound) were performed with each different incubation medium. Values are mean±SEM.
difference among the rates of lysis for clots that were exposed to t-PA that was in buffer, plasma, or buffer containing plasminogen ($p>0.05$). There was also no difference in lysis rates for clots that were incubated with these different supernatants in the presence of ultrasound, although a trend toward further improved thrombolysis with plasma and plasminogen compared with buffer is evident at 200 minutes (Table 1).

**Effect of Insonation on t-PA-Induced Thrombolysis In Vivo**

Based on the in vitro studies, investigation of insonation effect on thrombolysis was extended in vivo using the rabbit jugular vein thrombosis model (Figure 5, Table 2). In addition to the paired groups of rabbits that received t-PA in the presence or absence of ultrasound, four rabbits received only ultrasound and no infusion of t-PA. In these four controls, clot lysis was 9±5% at 50 minutes and 6±10% at 100 minutes (Table 2).

Lysis in the rabbits that received 1 mg t-PA and ultrasound was increased at 50 minutes compared with that of rabbits receiving t-PA alone (41±14% versus 20±12%, $p=0.12$). The increase at 100 minutes, a time when lysis had reached a plateau, was sustained (55±11% versus 30±12%, $p=0.11$). Furthermore, four of six pairs showed improved thrombolysis with ultrasound.

Clot lysis for the rabbits that received 2 mg t-PA and insonation compared with those that received only t-PA was 45±7% versus 28±11% ($p=0.10$) at 50 minutes and 50±12% versus 40±12% ($p=0.29$) at 100 minutes. Three of the five pairs showed improved thrombolysis with ultrasound.

Although the overall difference (repeated-measures ANOVA using data at 50 and 100 minutes for both t-PA doses) in mean thrombolysis between rabbits receiving insonation and t-PA and those receiving t-PA alone was not significant [$F(1,9)=2.7; p=0.13$], differences favored insonation at both 50 and 100 minutes for both doses of t-PA. The contrast between insonation and t-PA versus t-PA alone was greatest at 50 minutes [$F(1,9)=3.45; p=0.09$] when the combined data for the two doses were analyzed. The extent of thrombolysis in the group receiving t-PA at a dose of 2 mg was not different from that in the group receiving 1 mg ($p=0.73$).

The rabbits receiving 2 mg t-PA alone showed no significant change in the levels of plasminogen and fibrinogen between the beginning and the end of the experiment at 200 minutes. Fibrinogen levels were 2.2±0.2 mg/ml initially and then 1.6±0.3 mg/ml ($p=0.13$) at 200 minutes. Plasminogen levels were 3.9±0.3 CTA units/ml and then 3.7±0.2 CTA units/ml ($p=0.6$). The rabbits receiving ultrasound and t-PA showed significant changes in the levels of fibrinogen and plasminogen. The fibrinogen level decreased from 2.0±0.1 mg/ml at the beginning of the experiment to 1.1±0.3 mg/ml at 200 minutes ($p=0.03$). The plasminogen level decreased from 3.6±0.4 to 2.6±0.3 CTA units/ml ($p=0.03$). There was no significant change in the levels of fibrinogen or plasminogen in the four animals receiving ultrasound without t-PA. In this group, fibrinogen levels at the beginning and end of the experiment were 1.9±0.1 and 2.0±0.1 mg/ml ($p=0.42$), respectively, and the plasminogen levels were 4.2±0.3 and 4.0±0.2 CTA units/ml ($p=0.33$).

**Investigation of Potential Insonation-Induced Injury**

No increases in temperature were observed for animals receiving ultrasound and t-PA compared with those receiving only t-PA. There was no difference in the CPK values between rabbits receiving ultrasound and 1 mg t-PA and those receiving 1 mg t-PA alone. Pulmonary emboli were not detected histologically in 20 sections from the lungs of four rabbits treated only with t-PA. Two of the forty histological sections from eight rabbits that received combination t-PA and ultrasound

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**Table 2. Clot Lysis in Rabbits Receiving t-PA Alone or t-PA and Intermittent Ultrasound**

<table>
<thead>
<tr>
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<th>% Lysis at 50 minutes</th>
<th>% Lysis at 100 minutes</th>
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<tr>
<td></td>
<td>US</td>
<td>No US</td>
</tr>
<tr>
<td>t-PA (1 mg)</td>
<td>41±14</td>
<td>20±12</td>
</tr>
<tr>
<td>(n=6 pairs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-PA (2 mg)</td>
<td>45±7</td>
<td>28±11</td>
</tr>
<tr>
<td>(n=5 pairs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control rabbits:</td>
<td>9±5</td>
<td>ND</td>
</tr>
<tr>
<td>no t-PA</td>
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t-PA, tissue-type plasminogen activator; US, ultrasound; ND, not done; US frequency and intensity, 1 MHz and 1.75 W/cm². Values are mean±SEM.
had a single embolus, and each was located in a 1-mm arteriole.

Ultrasound did not induce endothelial changes in the rabbits that underwent a sham operation with catheterization of the jugular vein, and there was no evidence for thrombosis.

Discussion

The present study demonstrates that intermittent ultrasound (1 MHz, 1.75 W/cm²) can significantly enhance t-PA-induced fibrinolysis in vitro. This mode of ultrasound has minimal effect on clot lysis in the absence of t-PA. We postulate that ultrasound causes the clot to undergo more rapid fragmentation in the presence of t-PA, which allows increased binding of t-PA to newly exposed fibrin binding sites. Ultrasound could induce this effect through the process of acoustic streaming, which is the unidirectional movement of fluid in the ultrasound field.18 This causes large velocity gradients to develop at the boundary between fluid and more rigid structures, such as thrombi. Ultrasound can also induce biological effects through thermal injury18; however, temperature changes were not noted in any of the experiments in the present study that used pulsed ultrasound at a frequency of 1 MHz and intensity of 1.75 W/cm².

A third mechanism by which ultrasound can affect biological tissues is through cavitation, which is the activity resulting from a bubble or population of bubbles that are stimulated into action by an acoustic field.19 The intensity of ultrasound used in this study is below the range that can induce cavitation.13.18 However, this property has been used by other investigators to disrupt thrombi and atherosclerotic plaques.

In 1976, Trubestein and colleagues8 used a canine model of acute arterial and venous thrombosis to show that lysis could be induced by ultrasound applied through a catheter at the sites of the occlusions. This work was extended by Rosenschein and colleagues,10 who disrupted thrombi in vitro and in vivo in a canine model by delivering ultrasound intravascularly through a catheter that was placed in the clot. They demonstrated that ultrasound at the frequency of 20 kHz and power of ≥8 W induces its effects by cavitation13 and that tissues with high elastic content are relatively protected, whereas those with more rigid structures such as thrombi and atherosclerotic plaques are more easily destroyed.14 In a similar canine model, Ariani et al13 have shown that ultrasound can be delivered under angioscopic guidance.

Several reports have described the successful use of intravascular ultrasound to open occluded atherosclerotic vessels in animals20 and in human patients.12,13 Siegel et al21 have shown that partial patency provided by ultrasound can be further extended by the use of balloon angioplasty. The effect of this mode of ultrasound, which must be delivered through catheters to avoid damage to normal tissue,20 is cavitational as well as mechanical because of the vibration of the wire. Ultrasound has not yet been used to lyse thrombi in human patients.

The potential for the noninvasive use of ultrasound in conjunction with t-PA for the lysis of acute arterial occlusion has been demonstrated in a canine model in which both femoral arteries were occluded for 2 hours, followed by the infusion of t-PA at a concentration of 1 mg/kg.15–17 The application of transcutaneous continuous ultrasound (200 kHz) near the site of one of the occluded limbs in each animal was associated with an 80% decrease in the time required for recanalization.

The potential significance of noninvasive ultrasound delivered intermittently at a higher frequency is suggested by the present study. In the studies in vitro, intermittent ultrasound consistently shortened the time to 50% thrombolysis by one half. Furthermore, ultrasound increased the extent of fibrinolysis induced by t-PA (300 IU/ml) by as much as 30–50% in a buffer or plasma medium. Thus, under these experimental conditions, levels of plasminogen or inhibitors of plasmin or t-PA at concentrations found in normal plasma did not greatly influence the lysis of the clot once it had been formed. The major factors were the concentration of t-PA and whether or not the clot was exposed to ultrasound in addition to t-PA. Onundarson et al29 have also reported enhancement of t-PA–induced clot lysis in vitro by ultrasound delivered at a frequency of 1 MHz and an intensity as high as 8 W/cm². The acceleration of fibrinolysis was not caused by thermal effects of the ultrasound, and the pattern of fibrin degradation products produced by the combination of ultrasound and t-PA was not different from that of t-PA alone.

In the rabbit jugular vein thrombosis model, ultrasound showed a trend toward enhancement of t-PA–induced fibrinolysis. Histological studies of jugular veins exposed to ultrasound did not reveal any significant changes, and serum CPK values were not altered by insonation. The use of ultrasound did not appear to increase the risk for significant pulmonary emboli resulting from clot lysis. Animals that received 2 mg t-PA combined with ultrasound developed a significant decrease in the levels of fibrinogen, although this was not associated with increased clot lysis compared with the animals receiving only 1 mg t-PA and ultrasound. Additional studies will be needed to determine whether ultrasound has a systemic effect on fibrinogen levels when used in combination with higher doses of t-PA.

There was no difference in clot lysis, which was approximately 50% when measured at 100 minutes, between the groups of rabbits that received 1 or 2 mg t-PA in the absence of ultrasound. Collen et al19 found that clot lysis was approximately 80% when measured at 6 hours in rabbits that had received 1 mg t-PA during 4 hours. This difference can be explained by the studies of Clozel et al,23 who showed that the effect of a given dose of t-PA is dependent on the timing of the administration (i.e., bolus versus 4-hour infusion). Furthermore, doses of t-PA that are higher than 0.4 mg/kg do not induce a dose-dependent fibrinolytic response in the rabbit jugular vein thrombosis model.23

The time and duration of t-PA administration as well as the mode and frequency of ultrasound that are most efficacious in promoting clot lysis have yet to be established. The in vitro studies and the preliminary studies in the rabbit and the canine models, however, provide support for the potential use of noninvasive ultrasound to enhance t-PA–induced fibrinolysis in vivo.

Conclusions

This study demonstrates that intermittent ultrasound at a frequency of 1 MHz and intensity of 1.75 W/cm² can significantly enhance t-PA–induced fibrinolysis in vitro.
In a rabbit jugular vein thrombosis model, intermittent ultrasound administered during a 200-minute period showed a trend toward enhancement of t-PA–induced fibrinolysis without causing thermal changes or inducing tissue damage. The effect may be due to acoustic microstreaming, which could result in gentle perturbation of the clot thereby allowing increased binding of t-PA to fibrin.

Limitations

The in vitro studies clearly demonstrate a significant enhancement of t-PA–induced fibrinolysis by ultrasound. Larger studies will be needed to accurately compute statistical significance concerning the degree of enhancement of fibrinolysis in vivo and to determine whether the risk of pulmonary emboli is increased when ultrasound is used in conjunction with t-PA. The study would be enhanced by determining whether this mode of ultrasound can enhance t-PA activity that is being infused through a catheter near the site of a venous or arterial occlusion. Efficacy of thrombolysis needs to be determined for arterial clots and for those that have undergone aging. Additional modes of ultrasound should also be studied in conjunction with t-PA or other thrombolytic agents.

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