Ultrasound-Enhanced Thrombolytic Effect of Tissue Plasminogen Activator–Loaded Echogenic Liposomes in an In Vivo Rabbit Aorta Thrombus Model—Brief Report

Susan T. Laing, Melanie Moody, Beverly Smulevitz, Hyunggun Kim, Patrick Kee, Shaoling Huang, Christy K. Holland, David D. McPherson

Objective—Ultrasound enhances thrombolysis when combined with a thrombolytic and a contrast agent. This study aimed to evaluate the thrombolytic effect of our tissue plasminogen activator (tPA)–loaded echogenic liposomes (ELIP) in an in vivo clot model, with and without ultrasound treatment.

Methods and Results—The femoral arteries of New Zealand White rabbits (n=4 per group) were cannulated. The abdominal aortas were denuded, and thrombi were created using a solution of sodium ricinoleate plus thrombin. Rabbits were then randomly selected to receive tPA-loaded ELIP (200 µg of tPA/5 mg of lipid) or empty ELIP with or without pulsed (color) Doppler ultrasound (5.7 MHz) for 2 minutes. Thrombus was imaged and echogenicity analyzed before and after ELIP injection. Blood flow velocities were measured at baseline, after clot formation, and serially after treatment up to 15 minutes. tPA-loaded ELIP highlighted thrombus in the abdominal aorta more effectively than empty ELIP (P<0.05). Ultrasound enhanced the thrombolytic effect of tPA-loaded ELIP, resulting in earlier and more complete recanalization rates (P<0.001).

Conclusion—This study demonstrates effective highlighting of clots and thrombolytic effect of tPA-loaded ELIP in an in vivo rabbit aorta clot model. Doppler ultrasound treatment enhances this thrombolytic effect, resulting in earlier and more complete recanalization rates. (Arterioscler Thromb Vasc Biol. 2011;31:1357-1359.)

Key Words: arterial thrombosis ■ contrast echo ■ Doppler ultrasound ■ echocardiography ■ thrombolysis

Ultrasound-mediated thrombolysis is an active area of clinical investigation, and the addition of ultrasound contrast agents to augment ultrasound-mediated thrombolysis is a natural progression of this technique. We have reported on the use of echogenic liposomes (ELIP) not only as an ultrasound contrast agent but also as an agent for ultrasound-releasable directed thrombolytic therapy. We have demonstrated that loading of a thrombolytic agent, tissue plasminogen activator (tPA), into our ELIP resulted in retention of its echogenic properties, with effective highlighting of clots in vitro and in vivo. We previously demonstrated effective thrombolysis using tPA-loaded ELIP in an in vitro clot model. A short (2-minute) duration of low-frequency (1 MHz) continuous wave ultrasound treatment enhanced thrombolysis, with most of the effect likely related to tPA release from the ELIP. This study aimed to evaluate the thrombolytic effect of tPA-loaded ELIP with and without ultrasound treatment in an in vivo animal model of thrombosis.

Methods

Liposomal Formulation
ELIP were prepared by the sonicication-lyophilization-rehydration method as described previously. Recombinant tissue-plasminogen activator (tPA) (Activase Genentech Inc, San Francisco, CA) (400 µL of a 1 mg/mL tPA solution) was used for the initial rehydration of the lipid film. Entrapped tPA was separated from free tPA by centrifugation at 16 000 rpm for 10 minutes at 37°C. This results in a 50% encapsulation efficiency of tPA in the ELIP. Each aliquot is expected to contain 200 µg of tPA per 5 mg of lipid. Measurement of immunoreactive tPA in detergent-solubilized tPA-loaded ELIP using a quantitative sandwich enzyme-linked immunosorbent assay yielded a value of 156±49 µg of tPA per aliquot.

Animal Preparation
This study was approved by the Center of Animal Care and Use of the University of Texas Health Science Center, Houston. Male New Zealand White rabbits (3 to 4.5 kg) were anesthetized and surgically prepared. Small cutdown incisions were made in the neck and the inguinal area to place intrarterial sheaths into the right carotid and right femoral arteries. A Fogarty embolectomy catheter (2 to 4 French) was inserted through the femoral sheath and advanced into the proximal abdominal aorta under fluoroscopic guidance. The balloon was inflated until gentle resistance to pulling was felt, and the aortic endothelium was denuded by moving the catheter distally ~5 to 8 cm. With the balloon inflated, a 5% sodium ricinoleate solution (a sclerosing agent) combined with 1000 U of thrombin was administered through the central catheter lumen into the abdominal aorta to produce an occlusive thrombus. The balloon remained inflated for 2 minutes, after which it was deflated and the catheter was pulled back into the right femoral artery. A catheter was then
introduced through the carotid sheath and advanced under fluoroscopic guidance to the abdominal aorta just below the diaphragm and proximal to the aortic clot.

Thrombolysis Studies
Rabbits were randomly selected to receive saline (control), empty ELIP (5 mg lipid), or tPA-loaded ELIP (200 μg of tPA/5 mg of lipid). Treatments were delivered intraarterially proximal to the aortic thrombus. Rabbits were randomly selected to receive ultrasound treatment or not. Ultrasound treatment was delivered transabdominally over the aortic thrombus (5.7 MHz duplex [color] Doppler, mechanical index = 0.4, peak rarefractional pressure = 1.25 MPa, pulse repetitive frequency = 5 kHz) for 2 minutes using a clinically available ultrasound system (Vivid i with an 8L-RS linear array probe, GE Medical Systems, Milwaukee, WI). These ultrasound parameters have been shown to produce rapid fragmentation of ELIP with release of the encapsulated tPA.10 Ultrasound treatment was initiated once the tPA-loaded ELIP were visualized within the abdominal aorta.

Blood flow velocities were measured just above the aorto-iliac bifurcation distal to the aortic thrombus using pulsed Doppler ultrasound, at baseline, after clot formation, and serially every 2 minutes after treatment for up to 15 minutes. Results are reported as percentage of recanalization rate, which is defined as treatment blood flow velocity divided by baseline blood flow velocity.

Echogenicity Analysis
The aortic clot was imaged and echogenicity analyzed offline before and after injection of the tPA-loaded ELIP. Acoustic enhancement of the aortic clot was assessed by digitally recording B mode ultrasound images at 5 minutes after ELIP injection. Relative echogenicity (apparent brightness) of the aortic clot was quantified using a custom digital image analysis protocol. All image processing and analyses were performed with Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD). Data are reported as mean grayscale values.

Statistics
Multiple groups were compared using the analysis of variance with pairwise comparison performed using the Tukey test. The Kruskal-

Wallis analysis of variance on ranks was used for categorical variables. A probability value of <0.05 was considered significant. Analyses were performed using Sigma Stat 3.5 (Systat Software, Inc, Chicago, IL).

Results
tPA-loaded ELIP effectively highlighted thrombus in the rabbit abdominal aorta; this highlighting was significantly different from that of treatment with empty ELIP or saline (control) (Figure 1; \( P<0.05 \)).

There was no thrombolysis when animals were treated with saline (control), empty ELIP, or empty ELIP with ultrasound (\( P=\text{not significant} \)). Treatment of the thrombus with tPA-loaded ELIP resulted in recanalization of the abdominal aorta, as demonstrated by improvement in blood flow in the distal aorta (\( P<0.001 \) versus control and empty ELIP). Ultrasound application enhanced the thrombolytic effect of tPA-loaded ELIP, resulting in earlier and more complete recanalization rates (Figure 2; \( P<0.001 \) versus tPA-loaded ELIP alone and empty ELIP plus ultrasound).

Discussion
Improved thrombolysis with the concomitant use of focused ultrasound and a thrombolytic agent has recently been demonstrated for the treatment of acute thrombotic vascular diseases.1–4 For the clinical application of noninvasive ultrasound therapy, it may be advantageous to visualize the clot to selectively insonify the area of interest, thus limiting potential harmful bioeffects to surrounding tissues.

We have previously reported entrapment of tPA into ELIP with demonstration of effective highlighting of clots and thrombolysis in vitro.5 We also confirmed the high-affinity association of tPA with fibrin and demonstrated that this binding affinity was fully retained by the tPA-loaded ELIP.8
In this study, we demonstrated delivery of tPA-loaded ELIP to the thrombus site in an in vivo rabbit aorta model, as verified by real-time ultrasound imaging. This study was not designed to evaluate the effect of ultrasound alone on clot lysis but rather the effect of ultrasound on thrombolytic release from tPA-loaded ELIP. Visualization of the highlighted clot allowed for both spatially and temporally directed drug release using focused ultrasound application. In addition, this is an animal model of acute thrombosis and effects cannot be extrapolated to a chronic thrombosis model. Nevertheless, in this study, Doppler ultrasound treatment enhanced the thrombolytic effect of tPA-loaded ELIP, resulting in earlier and more complete recanalization rates. This study demonstrates the potential of a novel thrombolytic technique, combining a targeted thrombolytic-loaded contrast agent and ultrasound application, to enhance thrombolysis, with clinical applications for acute thrombotic processes.

Sources of Funding
This work was supported by National Institutes of Health Grants 2R01-HL074002, 2R01-NS047603, and 3R01-HL059586.

Disclosures
None.

References
Ultrasound-Enhanced Thrombolytic Effect of Tissue Plasminogen Activator–Loaded Echogenic Liposomes in an In Vivo Rabbit Aorta Thrombus Model—Brief Report
Susan T. Laing, Melanie Moody, Beverly Smulevitz, Hyunggun Kim, Patrick Kee, Shaoling Huang, Christy K. Holland and David D. McPherson

Arterioscler Thromb Vasc Biol. 2011;31:1357-1359; originally published online March 24, 2011;
doi: 10.1161/ATVBAHA.111.225938
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/6/1357

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/