Can Transcranial Ultrasonication Increase Recanalization Flow With Tissue Plasminogen Activator?
Toshihiro Ishibashi, MD; Masahiko Akiyama, MD; Hisashi Onoue, MD; Toshiaki Abe, MD; Hiroshi Furuhata, MD, PhD

Background and Purpose—In thrombolytic therapy for acute ischemic stroke, it is essential to obtain rapid thrombolysis before ischemic neuronal injury occurs. To develop a new technique of thrombolysis for acute ischemic stroke, the effect of transcranially applied ultrasound (TUS) on thrombolysis was examined.

Methods—An occlusion model of rabbit femoral artery was produced with thrombin after establishment of stenotic flow and endothelial damage. After stable occlusion was confirmed, monteplase (mtPA) was administered intravenously, and ultrasound (490 kHz, 0.13 W/cm²) was applied through a piece of temporal bone (TUS group; n=9). The control group received mtPA alone (tissue plasminogen activator [tPA] group; n=12). To verify the efficacy of TUS, femoral artery flow was measured during the procedure.

Results—The recanalization ratio was 16.7% (2 of 12) in the tPA group and 66.7% (6 of 9) in the TUS group. The recanalization ratio in the TUS group was higher than that in the tPA group (P=0.03). Patency flow ratio, which was defined as recanalization flow divided by baseline flow, of the TUS group (44.6±13.9%) was significantly greater than that of the tPA group (9.9±6.8%) at 60 minutes (P=0.025). Patency flow ratio became higher in the TUS group than in the tPA group between 20 and 30 minutes from the start of thrombolysis.

Conclusions—Low-frequency and low-intensity TUS enhanced thrombolysis by mtPA in a rabbit femoral artery occlusion model. This technique should be clinically useful for thrombolysis in acute ischemic stroke. (Stroke. 2002;33:1399-1404.)

Key Words: thrombolytic therapy ■ tissue plasminogen activator ■ ultrasonography, transcranial ■ rabbits

Current thrombolytic therapy for acute ischemic stroke in general involves the intravenous administration of thrombolytic agents or intra-arterial local fibrinolysis with the technique of radiological intervention. Most prospective clinical trials have been performed in acute ischemic stroke within 3 hours because delayed use of thrombolytic agents and delayed recanalization of major vessels may increase the risk of intracerebral hemorrhage. Moreover, recanalization rates by intravenous tissue plasminogen activator (tPA) range from 10% for internal carotid artery occlusion to 30% for middle cerebral artery occlusion. In the Prolyse in Acute Cerebral Thromboembolism (PROACT II) study, the recanalization rate was 66% for the recombinant prourokinase group. It has become an essential condition of the thrombolytic treatment of acute ischemic stroke that thrombolysis should be completed before ischemic neuronal injury occurs; therefore, it is important to dissolve the thrombus as early as possible.

In relation to thrombolytic therapy of myocardial infarction, there have been reports on the thrombolytic effects of ultrasound (US) both in vitro and in vivo (Table 1). US thrombolytic therapy with the use of an intravascular catheter has also been performed, which leads to earlier recanalization. We have previously reported that low-frequency, low-intensity US penetrates the cranium and enhances thrombolysis in vitro. This fact led us to begin to develop a new technique of thrombolytic therapy for acute ischemic stroke that would make possible thrombolysis of a cerebral embolism at an extremely early stage. Thrombolysis with transcranially applied US (TUS) has been investigated only in vitro experiments. This is the first in vivo study of thrombolysis with low-frequency, low-intensity TUS.

Materials and Methods

Animal Model
All animal procedures were performed under the guidance of the Animal Research Committee (Jikei University School of Medicine, Tokyo, Japan). Twenty-one New Zealand White rabbits weighing 2400 to 3200 g were used. The rabbits were anesthetized with intravenous thiopental sodium (20 mg/kg) via the marginal ear vein.

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TABLE 1. Characteristic of Published Reports of Thrombolysis With US

<table>
<thead>
<tr>
<th>Author</th>
<th>Protocol</th>
<th>Frequency</th>
<th>Output</th>
<th>Mode</th>
<th>Thrombolytic Agent</th>
<th>Thrombolysis Ratio*</th>
<th>Reflow Ratio or % Flow Ratio*</th>
<th>Sonication Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Francis19 (1992)</td>
<td>In vitro</td>
<td>1 MHz</td>
<td>1–8 W/cm²</td>
<td>CW</td>
<td>tPA</td>
<td>140% (1 W/cm²)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150% (2 W/cm²)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>178% (4 W/cm²)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>458% (8 W/cm²)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Lauer13 (1992)</td>
<td>In vitro</td>
<td>1 MHz</td>
<td>1.75 W/cm²</td>
<td>Interval</td>
<td>tPA</td>
<td>152%</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Luo14 (1993)</td>
<td>In vitro</td>
<td>1 MHz</td>
<td>1–2.2 W/cm²</td>
<td>CW</td>
<td>Streptokinase</td>
<td>55% (2.2 W/cm²)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Harpaz10 (1994)</td>
<td>In vitro</td>
<td>1 MHz</td>
<td>2.5 W/cm²</td>
<td>CW</td>
<td>tPA</td>
<td>514.50%</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Kimura11 (1994)</td>
<td>In vitro</td>
<td>300 kHz, 1 MHz</td>
<td>0.07, 0.4 W/cm²</td>
<td>CW</td>
<td>tPA</td>
<td>147% (tPA uptake)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Francis15 (1995)</td>
<td>In vitro</td>
<td>1 MHz</td>
<td>4 W/cm²</td>
<td>PW</td>
<td>tPA</td>
<td>193% (tPA uptake)</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Akiyama16 (1998)</td>
<td>In vitro</td>
<td>211.5 kHz, 1.03 MHz</td>
<td>0.25 W/cm²</td>
<td>CW</td>
<td>Urokinase</td>
<td>140%</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Suchkova17 (1998)</td>
<td>In vitro</td>
<td>40 kHz</td>
<td>0.25–1.5 W/cm²</td>
<td>CW</td>
<td>tPA</td>
<td>175% (0.25 W/cm²)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Behrens10 (1999)</td>
<td>In vitro</td>
<td>33.3, 71.4 kHz</td>
<td>0.5–3.4 W/cm²</td>
<td>tPA</td>
<td></td>
<td>130% (33 kHz)</td>
<td>60</td>
<td></td>
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<tr>
<td>Kudo18 (1989)</td>
<td>In vivo</td>
<td>200 kHz</td>
<td>tPA</td>
<td></td>
<td></td>
<td>400%</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Lauer13 (1992)</td>
<td>In vivo</td>
<td>1 MHz</td>
<td>1.75 W/cm²</td>
<td>Interval</td>
<td>tPA</td>
<td>916%</td>
<td>180</td>
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<tr>
<td>Kornowski12 (1994)</td>
<td>In vivo</td>
<td>1 MHz</td>
<td>6.3 W/cm²</td>
<td>Interval</td>
<td>tPA</td>
<td>120%</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Luo15 (1996)</td>
<td>In vivo</td>
<td>26 kHz</td>
<td>16 W/cm²</td>
<td>Streptokinase</td>
<td></td>
<td>983%</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Riggs17 (1997)</td>
<td>In vivo</td>
<td>1 MHz</td>
<td>2 W/cm²</td>
<td>Interval</td>
<td>Streptokinase</td>
<td>410%</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Luo16 (1998)</td>
<td>In vivo</td>
<td>37 kHz</td>
<td>160 W</td>
<td>PW</td>
<td>Streptokinase</td>
<td>1492%</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Suchkova18 (2000)</td>
<td>In vivo</td>
<td>40 kHz</td>
<td>0.75 W/cm²</td>
<td>CW</td>
<td>Streptokinase</td>
<td>1185%</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

CW indicates continuous wave; PW, pulsed wave; and interval, intermittent insonation.

*Compared with control results.

A tracheal intubation was performed, and the anesthesia was maintained with 1% to 1.5% isoflurane. Arterial blood pressure was monitored during the procedure. The animals were kept normothermic (38.0±0.3°C) by use of a heating blanket, and core body temperature with rectal digital thermometer was measured.

Thrombotic occlusion of the femoral artery was produced by an approach that has been described previously. An ultrasonic blood flowmeter (Transonic 1RB; Transonic) was set in place proximal to the femoral artery, and the peripheral end of a saphenous artery was ligated. Prestenotic flow was recorded via ultrasonic flowmeter (Transonic 1RB; Transonic) connected to a computer running PowerLab data acquisition software (Chart, ADI Instruments), and the arterial flow was monitored during the procedure. The animals were kept normothermic with 1% to 1.5% isoflurane. Arterial blood pressure was maintained with 1% to 1.5% isoflurane. Arterial blood pressure was monitored during the procedure. Stenosis was produced distal to the flow probe by constricting the artery with 5-0 silk suture to reduce the flow, and baseline flow was measured. Then a 1-cm segment of the femoral artery distal to the stenosis was clamped with temporary aneurysm clips, and the segment was traumatized by 3 external compressions with blunt forceps to produce endothelial injury. Then a polyethylene (PE50) catheter (Intramedic, Nippon Becton Dickinson Co Ltd) filled with 100 U/mL bovine thrombin (T-4648, Sigma Chemical Co) was inserted from the medial saphenous artery to just distal to the stenosis. To produce clots, 15 to 20 mL of blood was suctioned, and the blood was reintroduced with 1 U thrombin. Immediately after the PE50 catheter was withdrawn, the femoral artery distal to the stenosis was clamped. The proximal and distal clips were released 30 minutes later, and the absence of blood flow was monitored with the flow probe for 10 minutes to confirm a stable occlusion of the femoral artery. If the blood flow was not 0 mL/min at this time, additional clamping was performed for 10 minutes. All animals were observed for 10 minutes after the clamps were removed. The total age of the clots was 40 or 50 minutes. Each experiment was commenced after confirmation of zero flow.

**TUS System and Temperature Measurement**

The US system was described in our previous study. The automatic intermittent controller was installed as a new addition to the previous system. The US transducer used in this study was a ceramic type with a diameter of 10 mm, newly designed for animal studies. The transducer was connected to an amplifier, and an US generator (Honda Denshi Co) provided a continuous-wave output at 490.6 kHz. A human temporal bone (4 mm thick) was placed just below the transducer and wrapped in transparent kitchen plastic sheeting (Saran Wrap, Asahi Chemical) and used in a model example of TUS (Figure 1). US was applied with a simple cooling device to prevent heating of the temporal bone by an US transducer, which could affect the blood vessels. This device was made of a plastic container with transparent plastic sheeting adhering to its floor so as not to attenuate the US emissions. The container was filled with saline at room temperature, and the wrapped bone and the US transducer were inserted into it. The skin was not closed over the femoral artery. The incised edge of the operating field was drawn up with a silk thread, and the field was then filled with saline at room temperature. The TUS apparatus was placed in the water tank so that the end of its plastic container was 10 mm above the site of the embolism. The saline in the pool was exchanged when suitable to avoid temperature...
rise around the arteries. At the time of insonation, the temperature within the muscle surrounding the femoral artery was monitored continuously with a needle-type temperature sensor (PTW-100A, Unique Medical) and was taken as an indication of a rise in the temperature of the acoustic field of the transducer. We have already reported that low-frequency US has good penetration through the temporal bone. On the basis of that finding, we selected a frequency of 490.6 kHz for thrombolysis with TUS. The US intensity was determined with an Ultrasound Power Meter (UPM-DT-1; Ohmic Instruments Co). When the US intensity was set at 0.83 W/cm², the intensity through the temporal bone was 0.13 W/cm². We did not measure the spatial power distribution of US beam by hydrophone because the total intensity 0.83 W/cm², which equals approximately 0.011 MPa in water, is less than the threshold level of cavitation at 490.6 kHz. To avoid tissue heating, US was applied in cycles of 2 minutes with a 30-second pause for 10 minutes. Then US was not applied for 5 minutes. These 10-minute cycles, followed by 5-minute breaks, were repeated 4 times. Total intermittent application time of US was, with pauses, 60 minutes. Actual application time of US was 32 minutes. The blood flow and the intramuscular temperature were measured during the 30-second pauses, and these measurements were repeated every 2 minutes.

Study Protocol
After embolization was completed, monteplase (mtPA) (Clereacto, Eisai Co) was administered into the posterior auricular vein. Two groups were used: the TUS group received 1.2 mg mtPA with TUS, and the tPA group received 1.2 mg mtPA alone. TUS was performed simultaneously with the administration of mtPA, and the blood flow was measured continuously for 60 minutes. The blood flow and intramuscular temperature were recorded every 2 minutes. In this study, patency flow ratio was defined as recanalization flow divided by baseline flow. Recanalization was defined as an increase of at least 0.5 mL/min in the blood flowmeter reading, whether or not blood flow 60 minutes after the procedure was patent. Blood flow was measured every 2 minutes, and the aggregate over 60 minutes was taken as the flow volume. At the end of the experiment, cardiac arrest was induced in all of the animals by administration of an overdose of pentobarbital, and samples for histological examination were excised and fixed by 10% buffered formalin. The specimens were stained with hematoxylin and eosin.

Statistical Analysis
All statistical analyses were performed with the use of SAS software. All data were expressed as mean±SEM. Fisher’s exact test was used to compare the occurrence of recanalization between the 2 groups (Table 2). Statistical analysis of the patency flow ratio and basic parameters was performed by tailed t test (Table 3, Figure 2). The time course of patency flow ratio was analyzed by repeated-measures ANOVA (Figure 3). A value of P<0.05 was considered significant.

Results
Embolization of the femoral artery was performed in 21 rabbits with a mean body weight of 2809±25 g and without significant intergroup differences in weight. Their mean blood pressure during experiments was 66.5±2.3 mm Hg, and no significant intergroup difference was found in this variable. Mean rectal temperatures was 38.0±0.3°C. The mean baseline flow was 8.1±0.9 mL/min. The mean stenosis ratio in the tPA group was 21.1±2.3% and in the TUS group was 23.7±1.3% (Table 2), which was not significantly different between the 2 groups. The recanalization ratio was 16.7% (2 of 12) in the tPA group and 66.7% (6 of 9) in the TUS group. The recanalization ratio in the TUS group was thus higher than that in the tPA group (P=0.03) (Table 3). The patency flow ratio in the TUS group (44.6±13.9%) was significantly greater than that in the tPA group (9.9±6.8%) (P=0.025) (Figure 2). The patency flow ratio became signif-

### Table 2. Basic Parameters in TUS Group and tPA Group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight, g</th>
<th>Baseline flow, mL/min</th>
<th>Stenosis ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA</td>
<td>12</td>
<td>2858±45</td>
<td>7.0±1.0</td>
<td>20.1±2.3</td>
</tr>
<tr>
<td>TUS</td>
<td>9</td>
<td>2766±29</td>
<td>5.3±0.6</td>
<td>23.7±1.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Basic parameters showed no significant difference between the 2 groups.

### Table 3. Occurrence of Recanalization in TUS Group and tPA Group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Recanalization Ratio, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA</td>
<td>12</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>TUS</td>
<td>9</td>
<td>66.7</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Any recanalization (complete or partial flow improvement) was found at 60 minutes in 2 of 12 arteries in the tPA group and 6 of 9 in the TUS group. Recanalization ratio in the TUS groups was significantly higher than that in the tPA group (P=0.03).
Significantly higher with TUS between 52 and 60 minutes from the start of thrombolysis (Figure 3). Arteries that showed complete recanalization after TUS were found to be patent with small focal residual thrombus in light microscopic examination (Figure 4A [patent vessel] and 4B [partially patent vessel]). There were no additional endothelial injuries or histological changes in arteries to suggest damage by US exposure. Increase in intramuscular temperature was <3°C in the TUS group (Figure 5).

**Discussion**

The present study clearly demonstrated that low-frequency (490.6 kHz) and low-intensity (0.13 W/cm²) US, which was applied through the human temporal bone, significantly enhanced thrombolysis by mtPA in a rabbit femoral artery occlusion model. This in vivo study on thrombolysis in TUS extends previously performed in vitro studies. Various thrombolytic therapies, including intravenous administration of tPA and intra-arterial application of prourokinase, have been reported for acute ischemic stroke. Delayed thrombolysis causes irreversible neuronal injury and carries risk of hemorrhagic complications. This study sought to develop a prompt and less invasive therapy for acute ischemic stroke.

We modified the femoral artery occlusion model, which consisted of stenosis with endothelial injury and...
autologous thrombus. Justification of animal models of acute ischemic stroke should be evaluated on the basis of stenosis, endothelial injury, and thrombus formation. Stenosis of arteries is the fundamental requirement of a cerebral embolism model. The stenosis in this study (mean value of 24.3% stenosis by flow volume) was suitable for an experimental occlusion model based on the hemodynamic theory.26,27 Endothelial injury is also essential to thrombus formation, and therefore we injured the endothelium to lodge the autologous thrombus, which was injected externally in the femoral artery.

Various frequencies have been used for thrombolysis with US; the majority of studies report the use of 1 MHz (Table 1). Our selection of low-frequency (490 kHz) US was based on the following considerations. First, low-frequency US can penetrate the skull well and has the ability to accelerate thrombolysis by urokinase.7 Because tissue penetration declines at higher-frequency US, lower-frequency US is ideal for use in TUS thrombolytic therapy. Second, we sought to avoid adverse effects on the brain tissue. The mechanical effect of US is proportional to peak rarefractional pressure but is inversely proportional to US frequency.24 In addition, it is generally believed that the mechanical biological effect has a threshold according to which no effect occurs unless a certain output level is exceeded.25 The relation between rarefractional pressure and US frequency is defined as $p/\omega f=\text{constant}$, where $p$ is rarefractional pressure and $f$ is frequency. Furthermore, the mechanical effect of US has a threshold according to which no effect occurs under a certain output level.25 On the basis of this equation, the rarefractional pressure due to low frequencies, such as 30 and 70 kHz used in a previous study,8 is approximately 3 times that which occurs at the 490.6-kHz frequency. High rarefractional pressure may cause adverse effects such as cavitation.25 Additionally, we selected the intensity (0.13 W/cm²) to be as low as possible to avoid the mechanical biological effect and to dissolve thrombus by minimal invasion.

In addition to the mechanical biological effect, the thermal biological effect caused by the following 2 phenomena should also be considered an adverse effect. The first phenomenon is the temperature rise in the brain tissue and the skull by the US absorption phenomenon. According to the standard of the American Institute of Ultrasound in Medicine,25 the degree of thermal biological effect can be evaluated by the thermal index (TI), which indicates the temperature increase per unit volume of human tissue. In our experimental conditions of 490.6 KHz and intermittent application of US at 0.83 W/cm², TI in the brain tissue was estimated at 1.01 for 0.13 W/cm², and TI in the cranium was 2.08 for 0.83 W/cm²; these parameters were chosen to avoid the thermal adverse effect in the clinical application. Since the value of TI in soft tissue increases proportionally with frequency increase, the thermal effect can be decreased in lower-frequency US. Therefore, it is possible, in the interest of safety, to choose an optimal US frequency range that can avoid a large thermal effect in higher-frequency US and a large mechanical effect in lower-frequency US under the same power level. The other thermal effect is caused by the US transducer surface, which induced the energy loss in the transducer. By the use of a simple cooling system, our system succeeded in restricting the temperature rise to <3°C in the muscle surrounding the femoral artery, as shown in Figure 5. However, since this temperature increase was not enough to avoid this adverse thermal effect completely, a more precise cooling system should be used between the 2 surfaces of human temporal bone and the US probe in clinical practice.

The mechanism underlying enhanced thrombolysis by US is not fully understood. Because US alone does not enhance thrombolysis, as reported previously,14 it is thought that the enhancing effect of US on thrombolysis is not caused by direct destruction of the thrombus but rather by accelerated transport of thrombolytic agents. Several studies have suggested that permeation and disaggregation of fibrin fibers contribute to this mechanism.28–31 It has been verified from many in vitro studies that the thrombolysis ratio rises to approximately 1.5 times that obtained without US. In contrast, most of the in vivo studies have demonstrated that the enhancing effect is markedly stronger than that observed with the in vitro studies (Table 1). Our results also showed much greater enhancement of thrombolysis: when US was applied with rtPA, the recanalization ratio increased 5 times, and the patency ratio after 60 minutes was 20 times greater than that with rtPA alone. These differences between in vitro and in vivo results may be caused not only by direct thrombolytic effect but also by other related factors, such as endothelial function, platelet activation, and altered metabolism.32

In considering therapeutic applications for acute ischemic stroke, it is important to choose optimum US frequency, intensity, and sonication methods. In regard to tPA used in clinical practice, Alexandrov et al33 recently reported that patients in whom recanalization was monitored with transcranial Doppler had better outcome than those in whom transcranial Doppler was not used, and they reported the possibility of facilitated thrombolysis with combined tPA and US. However, 2-MHz and 0.2-W US in transcranial Doppler should be markedly attenuated to <5% through the cranium,24 and there has been no direct evidence for the enhancing effect of US at megahertz frequency on thrombolysis. One critical problem to be addressed is that of the limitation of TUS treatment in terms of safety. Further studies are needed to examine the ischemic brain to optimize US frequency and intensity as well as to develop new US equipment with a cooling system acceptable for clinical use.

We conclude that low-frequency, low-intensity US enhances thrombolysis with rtPA and improves blood perfusion. This method might be promising for shortening the period of recanalization and reducing the risk of hemorrhagic complications.

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References


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