Ultrasound Accelerates Thrombolysis of Acutely Induced Platelet-Rich Thrombi Similar to Those in Acute Myocardial Infarction

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Background Although sonothrombolysis has been studied for development of recanalization that is safer and more efficacious than the methods currently used, there have been no studies of the efficacy of sonothrombolysis for the platelet-rich thrombi that typically cause acute myocardial infarction (AMI). The effects of adding ultrasound (US) to pharmacological lysis of platelet-rich thrombi was examined in a rabbit model of femoral artery occlusion.

Methods and Results In 35 rabbits, the right femoral artery was balloon-injured repeatedly at 4-week intervals to induce platelet-rich thrombi. Two hours after the induction of occlusive thrombi, 27,500 IU/kg tissue plasminogen activator (tPA) were injected via an ear vein, with or without transcutaneous US (continuous wave, 1 MHz, 0.75 W/cm²), or 13,750 IU/kg tPA was administered with US (n=10). Significantly higher rates of successful thrombolysis (Thrombolysis In Myocardial Infarction grade 3) were observed with US (90.0%) than without it (10.0%), irrespective of the dose of tPA used (p<0.01). The peak flow velocity in affected femoral arteries was significantly higher with US (p<0.01), and histological examination confirmed complete dissolution of thrombi. However, the thrombi were not affected by US alone (n=5).

Conclusions US facilitates thrombolysis of platelet-rich thrombi and could be a useful component of thrombolytic therapy following AMI. (Circ J 2007; 71: 1643–1648)

Key Words: Myocardial infarction; Plasminogen activator; Platelets; Thrombolysis; Ultrasound

The prognosis of acute myocardial infarction (AMI) is greatly influenced by the time to recanalization after onset; the sooner the occluded coronary artery is reperfused, the smaller the infarct and the greater the preservation of cardiac function. Thus, the development of recanalization therapies that are faster and safer than the ones currently in use should provide better prognoses for AMI patients.

Current recanalization therapy includes pharmacological thrombolysis and percutaneous coronary intervention (PCI), both of which have improved the prognoses of AMI patients!^{1–4} In their current forms, however, each of these therapies has its limitations. For instance, PCI is time consuming and expensive, requiring transportation of patients to a specialized hospital at which cardiac catheterization can only be carried out by highly trained medical staff. On the other hand, intravenous pharmacological thrombolysis is noninvasive, less expensive and simpler than PCI, and can be started just after diagnosis of AMI, even in an ambulance. With this approach, however, Thrombolysis In Myocardial

Infarction (TIMI) grade 3 flow is achieved in only 60–75% of patients^{5–8} Although a better reperfusion rate could be gained with higher doses of fibrinolytic agents, increasing the dose also makes hemorrhagic complications more likely. The next generation of thrombolytic therapies should be more rapid and produce higher rates of successful recanalization to TIMI grade 3 flow.

Following the first report of using ultrasound (US) to induce thrombolysis,⁹ many investigators began combining US with thrombolytic agents in an effort to enhance their efficacy!^{10–21} It was found that US enables the dose of thrombolytic agent to be reduced and shortens the reperfusion time in experimental animals. Thus, a combination of US and a thrombolytic agent, such as tissue plasminogen activator (tPA), appears to be a promising approach to thrombolytic therapy. In those earlier studies, however, the thrombolytic effects of US were investigated using fibrin-rich thrombi, which are quite different from those responsible for AMI. In AMI, tissue factor-mediated platelet aggregation plays the crucial role in thrombogenesis;^{22,23} consequently, the thrombi contain not only fibrin but also large numbers of platelets.^{22,24} Therefore, to confirm the utility of US for lysis of platelet-rich thrombi, in vivo, we recently established a rabbit model of femoral artery occlusion in which platelet-rich arterial thrombi are acutely induced by repeated balloon injury.^{25,26} We can then test whether US can enhance the pharmacological lysis of platelet-rich thrombi.

(Received March 26, 2007; revised manuscript received June 7, 2007; accepted June 18, 2007)

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Fig 1. Representative photographs obtained during angiography prior to (A) and 60 min after (B–D) the start of thrombolysis. Arrows show the occlusion site within the right femoral artery, and arrowheads show the enhanced end of the artery 60 min after tissue plasminogen activator (tPA) injection. In this case, Thrombolysis In Myocardial Infarction (TIMI) grade 0 flow was seen in the tPA-high-alone animal (B) and in the ultrasound (US) alone animal (D), whereas TIMI grade 3 flow was seen in the tPA-high+US animal (C).

Table 1 Angiographic Parameters

	tPA-high alone	tPA-high + US	tPA-low + US
<i>TIMI grade</i>			
0	n=3 (30%)		
1	n=2 (20%)		
2	n=4 (40%)	n=1 (10%)	n=1 (10%)
3	n=1 (10%)	n=9 (90%)*	n=9 (90%)*
Mean TIMI grade	1.30±0.34	2.90±0.10*	2.90±0.10*

tPA, tissue plasminogen activator; US, ultrasound; TIMI, Thrombolysis In Myocardial Infarction.

* $p < 0.01$ vs tPA-high alone group; $n=10$.

Methods

Rabbit Model of Acute Coronary Syndrome-Like Thrombus

The Animal Experimentation Committee at Nara Medical University approved the experimental protocol. We used 35 male Japanese white rabbits (SLC Japan, Shizuoka, Japan) weighing 2.5–2.7 kg in this study. On 2 occasions separated by a 4-week interval, the rabbits were anesthetized with pentobarbital sodium (25 mg/kg body weight, iv) and balloon injury of the right femoral artery was performed as previously described, with minor modification.^{25,26} Briefly, the first injury was induced by fluoroscopically inserting a PTCA balloon catheter (3.0 mm-diameter, 8.0 mm-length) via the left carotid artery to a distal site in the right femoral artery where it was inflated to 8 atm. The inflated balloon was then pulled back a distance of 3 cm 3 times. Four weeks later, formation of a stenotic lesion at the site of injury was confirmed angiographically, and then the second injury was induced. In this case, another PTCA balloon catheter (2.75 mm-diameter, 15 mm-length) was inserted via the right carotid artery to a site just distal to the stenotic

lesion, and a second injury was induced in the same manner as the first. Thereafter, blood flow in the injured right femoral artery was reduced to 10% by incomplete ligation distal to the injured area according to a transit time blood flowmeter (Transonic Systems Inc, NY, USA), which caused immediate formation of an occlusive thrombus.²⁶ To confirm the thrombotic occlusion, angiography was carried out using a catheter inserted into the abdominal aorta 15 min and 2 h after the second injury. Total occlusion of the right femoral artery was confirmed in all rabbits.

Thrombolytic Methods

Immediately after confirmation of occlusion of the right femoral artery, thrombolysis was initiated using tPA with or without US. The rabbits were assigned to 1 of 3 groups: (1) tPA-high-alone group received 27,500 IU/kg tPA, which corresponds to the dose used clinically in humans, without US; (2) tPA-high+US group received the same dose of tPA plus US; and (3) tPA-low+US group received 13,750 IU/kg tPA plus US ($n=10$ in each group). The bolus of tPA was administered via an ear vein in all groups and in the 2 sonothrombolysis groups US (continuous wave, 1 MHz, 0.75 W/cm² at the surface) was simultaneously applied transcutaneously over the thrombus up to 60 min until successful thrombolysis (TIMI grade 3) was obtained. Additionally, in another 5 rabbits (the US alone group), US was applied without tPA administration in order to investigate the direct effect of US on the platelet-rich thrombus. The US device comprised a cylindrical probe, 3 cm in diameter and 6 cm in length connected to a power amplifier.

Evaluation of Thrombolytic Effect

The effects of thrombolysis and sonothrombolysis were evaluated angiographically based on peak flow velocity.

Fig 2. Mean Thrombolysis In Myocardial Infarction (TIMI) flow grade for each group (* $p < 0.01$ vs tissue plasminogen activator (tPA)-high-alone group). Time course of thrombolysis following tPA injection, with and without ultrasound (US). Data are expressed as the percentage of rabbits in each group showing TIMI grade 3 flow (* and # $p < 0.01$ vs tPA-high-alone group).

Angiography was carried out every 15 min up to 60 min after the start of thrombolysis, and the TIMI flow grade was determined. In addition, 60 min after the tPA injection, the peak flow velocities were measured just proximal to the site of the injury in the right femoral arteries and at the same site in the left femoral arteries of rabbits with a TIMI flow grade of 2 or 3 using a Doppler Guide Wire connected to a FloMap system (Cardiometrics, Inc, CA, USA). Nitroglycerine (0.2 mg/min) was slowly injected for 1 min through the catheter located in the abdominal aorta just before angiography and the measurement of the peak flow velocity to avoid the influence of vasospasm. The peak flow velocity of the affected femoral artery was expressed as a percentage of that in the contralateral control artery in each rabbit. In all rabbits, the affected femoral artery was harvested after the last angiography and fixed in 4% paraformaldehyde for 24 h at 4°C. The fixed arteries were then embedded in paraffin, cut into 3- μ m-thick cross-sections and stained with hematoxylin–eosin for histological evaluation.

Statistical Analysis

The data are expressed as means $\pm$ SD. Differences between individual groups were evaluated using unpaired Student t-test or Fisher's exact test. Values of $p < 0.05$ were considered significant.

Results

Angiographic Data

The repeated balloon injury caused complete thrombotic occlusion in the affected femoral artery in all rabbits (Fig 1A). Moreover, the affected femoral arteries in 9 of the 10 rabbits in the tPA-high-alone group were not fully recanalized 60 min after tPA administration (Fig 1B). By contrast, apparently complete recanalization was achieved in 9 of 10 rabbits in both the tPA-high + US and tPA-low + US groups (Fig 1C).

The rates of angiographic patency (TIMI grade 3 flow) in the tPA-high + US and tPA-low + US groups were identical, and significantly higher than in the tPA-high-alone group, at 60 min after tPA administration (Table 1). In the tPA-high-alone group, TIMI 3 flow was obtained in only 1 case (10%), whereas persistent occlusion (TIMI 0 flow) was seen in 3 cases (30%). Both groups receiving tPA + US had a greater frequency of complete reperfusion: TIMI 3

in 9 cases (90%) and TIMI 2 in 1 case (10%) in both the tPA-high + US and the tPA-low + US groups. The mean TIMI flow grade was also significantly higher in both the tPA-high + US (2.90 ± 0.10) and the tPA-low + US (2.90 ± 0.10) group than in the tPA-high-alone group (1.30 ± 0.34) (Table 1; Fig 2A).

Fig 2B shows the time course of thrombolysis in each group. Whereas only 1 rabbit (10%) receiving tPA alone showed TIMI grade 3 flow in the affected femoral artery within 60 min, 9 rabbits (90%) receiving US and either dose of tPA showed TIMI grade 3 flow within 30 min.

On the other hand, no obvious angiographic change in the occlusive thrombus was observed in all rabbits in the US alone group (Fig 1D), which suggests that transcutaneous US itself has no distinct effect on platelet-rich thrombi in vivo.

Peak Flow Velocity

Peak flow velocity in the femoral artery was measured in rabbits with a TIMI flow grade of 2 or 3 ($n = 5$ in each group) after the last angiography. As shown in Fig 3, the peak flow velocities in the affected femoral arteries of rabbits receiving tPA + US (54.0 ± 3.9 cm/s in the tPA-high + US group and 53.3 ± 4.9 cm/s in the tPA-low + US group) were similar to those in the contralateral control arteries (55.7 ± 4.8 cm/s and 55.5 ± 4.8 cm/s, respectively). In the tPA-high + US group, peak flow velocity was $98.6 \pm 0.7\%$ of control, and in the tPA-low + US group, it was $97.4 \pm 0.6\%$ of control. By contrast, the peak flow velocities in the affected femoral arteries of rabbits not receiving US (41.7 ± 5.4 cm/s) were significantly slower than in the contralateral control arteries (54.7 ± 7.1 cm/s). In the tPA-high-alone group peak flow velocity was only $75.6 \pm 1.2\%$ of the control value.

Taken together, our angiographic and peak flow velocity data clearly show that US not only accelerates pharmacological thrombolysis, but also enables a lower dose of thrombolytic agent to be used.

Histology

Histological examination confirmed that thrombi were completely dissolved in rabbits receiving US with tPA, whereas substantial remnants of the thrombi remained in the affected femoral arteries of rabbits not receiving US (Fig 4). In addition, any remarkable change of the occlusive thrombus, such as fragmentation, was not observed in the rabbits

Fig 3. Representative images of peak flow velocity 60 min after tissue plasminogen activator (tPA) injection. In the tPA-high-alone group, the peak flow velocity in the affected femoral artery (a) is slower than in the contralateral control femoral artery (b). On the other hand, in the tPA-high+ultrasound (US) group, the peak flow velocity in the affected femoral artery (c) is similar to that in the contralateral control artery (d). Mean peak flow velocity in the affected femoral artery expressed as a percentage of that in the contralateral control femoral artery 60 min after tPA injection (* $p < 0.01$ vs tPA-high-alone group).

receiving US alone. These data suggested that transcatheter US could enhance thrombolysis with thrombolytic agents, but might not have a direct effect on platelet-rich thrombi.

Discussion

Our findings in a rabbit model of acute arterial occlusion, demonstrate that transcutaneous US accelerates pharmaco-

logical lysis of platelet-rich thrombi similar to those responsible for AMI. Sonothrombolysis led to complete recanalization of 90% of the affected arteries within 30 min, and after treatment the peak flow velocity in the affected arteries was same as in the contralateral control artery. These findings provide the first experimental evidence that US could serve as the basis of the next generation of thrombolytic therapies used in the treatment of acutely induced arterial thrombotic disease, including AMI.

Fig 4. Photomicrographs of representative right femoral arteries showing the thrombotic occlusion just prior to tissue plasminogen activator (tPA) injection (A), remnant thrombus in the artery of a tPA-high-alone animal (B) and absence of a thrombus in the artery of a tPA-high+ultrasound (US) animal (C) 60 min after tPA injection. In the US alone animal (D), no obvious change of the occlusive thrombus can be seen.

Over the course of the past decade, efforts have been made develop techniques for rapid recanalization after AMI that are safer and less expensive than catheter-based techniques. Most often, these methods have been studied in vitro using exogenously formed whole-blood clots, or in vivo using animal models of thrombosis.^{10–20} Although there have been several reports that US enhances thrombolysis, clinical application of sonothrombolysis in cases of AMI is not yet widely accepted. One reason for this is that many cardiologists consider the data from earlier studies insufficient to justify its clinical use, in part because the efficacy of sonothrombolysis has been demonstrated only with fibrin-rich thrombi, not the platelet-rich thrombi typically seen with AMI. In the present study, therefore, we tested the efficacy of sonothrombolysis in a rabbit model of femoral artery occlusion in which formation of platelet-rich thrombi was induced by repeated balloon injury.^{25,26} Our results demonstrate unequivocally that US enhances thrombolysis of platelet-rich thrombi.

In this study, we used US with a frequency of 1 MHz and an intensity of 0.75 W/cm², for 2 reasons. First, in earlier reports showing that US enhanced pharmacological thrombolysis, the frequency used was primarily 1 MHz.^{16,20,27,28} Second, as a result of direct transmission of energy and acoustic cavitation, high-intensity, low-frequency US breaks clots into small fragments,²⁹ which could then induce distal embolization. Although several investigators have recently shown that low-frequency US (20–40 kHz) provides better tissue penetration of thrombolytic agents than high-frequency US,^{17–19,21} the present study results clearly show that 1 MHz and 0.75 W/cm² US significantly enhanced tPA-induced thrombolysis. However, the mechanisms by which US accelerates thrombolysis have not been fully elucidated. At low intensity levels US exerts a “microstreaming” effect, which promotes the motion of fluids around the surface of thrombus. This microstreaming might

enhance the permeation of thrombolytic agents into the fibrin network. Several in vitro studies suggest that US directly changes the fibrin structure, inducing the better penetration of tPA into the thrombus.^{30,31} In the present study, we demonstrated that US enhanced the pharmacological lysis of platelet-rich thrombus, probably without direct influence on the structure of thrombus, indicated by the angiographic and histological data for the US alone group. Therefore, we consider that US accelerates the mixing of tPA into the thrombus in vivo by microstreaming. Moreover, our finding that the peak flow velocity of recanalized arteries following sonothrombolysis was the same as that in the contralateral control arteries indicates that there was no distal embolization by fragmented thrombi, which was supported by the data for the US alone group. We therefore conclude that 1 MHz and 0.75 W/cm² US was both safe and effective for sonothrombolysis in the present femoral artery model.

There have been several recent clinical reports showing the beneficial effect of combining US with tPA in cases of acute ischemic stroke.^{32–34} On the other hand, 1 clinical study of the efficacy of sonothrombolysis in AMI found that TIMI grade 3 flow was achieved in only 64% of patients treated with both US and a fibrinolytic agent³⁵ which is approximately the same rate previously reported for pharmacological thrombolysis without US.^{5–8} The reason why clinical findings show US to be promising for potentiation of thrombolysis in stroke but not AMI is unclear at present. One possibility is that there is a difference in the nature of the thrombi formed in stroke and AMI: approximately 50% of thrombi formed in strokes are fibrin-rich, whereas almost all thrombi formed in AMI are platelet-rich. However, that possibility is not consistent with our findings, which indicate that US significantly potentiates pharmacological lysis of platelet-rich thrombi. Another possibility is that it is difficult to focus the US on the coro-

nary thrombus because of the cyclic movement of the coronary artery and the limited window for US application. An improved US device that would enable the beam to be applied from the narrow intercostal space and aimed at a mobile target, could remedy this situation.

In summary, we have shown for the first time that US can facilitate pharmacological lysis of platelet-rich thrombi, such as those responsible for AMI, and reduce the dose of thrombolytic agent needed. Although we demonstrated the efficacy of sonothrombolysis of platelet-rich thrombi in a model of femoral artery occlusion model, our findings suggest the possible benefit of using transcutaneous US to facilitate arterial recanalization following AMI.

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