Evaluation of the Thrombolytic Effect of Tissue-Type Plasminogen Activator with Ultrasonic Irradiation: *In Vitro* Experiment Involving Assay of the Fibrin Degradation Products from the Clot

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Previous studies show that the combined method using thrombolytic drugs and ultrasonic irradiation enhances the effect of thrombolysis in *in vitro* and *in vivo* experiments.

However, the mechanism of the clot lysis was unclear. In previous studies the evaluation of the clot lysis effect was determined by the rate of decrease in clot weight or the decrease in radioisotope-labeled fibrinogen in the clot. However, it was unclear whether or not the fibrinogen in the clot was degraded.

We measured quantitatively the fibrin degradation product, D-dimer (FDP-DD), produced from clots subjected to recombinant tissue-type plasminogen activator (rt-PA, 2.0 $\mu\text{g/ml}$), with and without ultrasonic irradiation.

Two levels of continuous ultrasound were used, 300 kHz (100 V, 0.07 W/cm²) and 1 MHz (20 V, 0.4 W/cm²). The quantity of FDP-DD produced was 1.74 times at 300 kHz and 1.80 times at 1 MHz, greater than that measured in rt-PA solution without ultrasonic irradiation; clot lysis was not observed in normal saline with ultrasonic irradiation. Our experiment clarifies that the mechanism of clot lysis when subjected to a combination of drug and ultrasound leads to degradation of fibrin, allowing a quantitative measurement of the enhancement of clot lysis. A high correlation was observed between the FDP-DD produced from the clots and the rate of decrease in clot weight.

Keywords thrombolysis; ultrasound; recombinant tissue-type plasminogen activator (rt-PA); fibrin degradation product (FDP)

Recent thrombolytic therapy for acute myocardial infarction has included the use of urokinase, tissue-type plasminogen activator (t-PA) and other drugs.¹⁻⁴⁾ Although these drugs have demonstrated a specific thrombolytic effect, such drug-based therapies are accompanied by undesired side effects involving bleeding from other organs⁵⁻⁸⁾ which is often dose dependent. In our study, a new thrombolytic approach, combining ultrasonic irradiation with standard drug treatment, has been examined with the aim of identifying the process involved in clot lysis by measuring the amount of fibrin degradation product, D-dimer (FDP-DD), produced in *in vitro* experiments. Previous studies have shown that this combined approach enhanced the thrombolytic effect of t-PA.⁹⁻¹²⁾ Kudo¹¹⁾ reported that in *in vivo* the combined method enhanced the thrombolytic effect and noted a decrease in the recanalization time to 40% and a decrease in the dose of drug required to 30%. However, previous studies have not examined the amount of FDP produced in the clot lysis process or even discovered whether FDP was produced by the combined drug-ultrasound approach.

MATERIALS AND METHODS

A standard clot was produced from fresh, whole, human blood, then clot lysis was examined in the four separate solutions: 1) normal saline, 2) normal saline with ultrasonic irradiation, 3) recombinant tissue-type plasminogen activator (rt-PA, Mochida Pharmaceutical Co., Ltd.) solution (2.0 $\mu\text{g/ml}$, in normal saline) and 4) rt-PA solution with ultrasonic irradiation. Groups 1, 2 and 3 served as controls. We evaluated the lysing effect on the clot by quantitative measurement of the residual

clot weight and FDP-DD.

Producing Blood Clots We sought a suitable procedure for making a standard clot by changing both the blood coagulating and incubation times. Glass tubes (inside diameter: 10 mm, length: 30 mm) was stood on glass plates. Silicone grease (H.V.G., Dow Corning-Toray Co., Ltd.) was put on the bottom of the glass tubes to prevent blood oozing from the tube on to the plate. Fresh human blood (0.8 ml) was poured slowly into each tube and 0.4 ml of liquid paraffin was laid over the blood to exclude air. For blood coagulation, the tubes were kept at 37 °C for between 1 and 5 h. Each clot was then placed in 20 ml of normal saline (about 15 °C) and the saline then removed by decantation. This procedure was repeated 3 or 4 times until floating blood cells could not be seen. The water on the clots was removed with absorbent paper. The clot weight was measured with an electronic balance (ED-H 200, Shimadzu Corporation, =W1). Then, the clots were replaced in 20 ml of normal saline and incubated at 37 °C while vibrating at 60 shakes/min for 1, 2 and 3 h. The clot weight was measured again (=W2). The decrease in the clot weight was calculated from W1 and W2 as the clot lysis ratio in normal saline. The clot incubated for 4 h was selected as the standard clot. Discussion of this selection can be found in the Result section.

rt-PA Concentration and Reaction Time After recording the initial weight (W1), the standard clot was placed in 20 ml of rt-PA solution and incubated at 37 °C while vibrating at 60 shakes/min. The rt-PA concentration was changed from 1.0 to 16.0 $\mu\text{g/ml}$ by successive doubling in order to identify a suitable concentration of rt-PA. Reaction times of 0.5, 1.0 and 2.0 h were studied. The decreasing ratio of the clot weights and the FDP-DD

Fig. 4. FDP-DD Level Produced from Clots and the Decrease in Clot Weight

The clots were incubated with $2.0 \mu\text{g/ml}$ rt-PA for 0.5, 1 and 2 h at 37°C . Each point and bar represent the mean and S.E. from 9 clots.

Fig. 5. Effect of Ultrasonic Irradiation on Clot Lysis

Results are expressed as the mean $\pm$ S.E.

not increase significantly. Neither the clot lysis rate nor the FDP-DD concentration exhibited a statistically significant increase with incubation time (Fig. 4).

Therefore, we decided that the rt-PA concentration should be set at $2.0 \mu\text{g/ml}$ and the incubation time should be 1 h.

Effect of Ultrasonic Irradiation Figure 5 shows the FDP-DD concentration and clot lysis under six different sets of conditions.

In normal saline, the FDP-DD concentration was $154 \pm 2 \text{ ng/ml}$ and the clot lysis was $13.4 \pm 3.2\%$ without ultrasonic irradiation. With ultrasonic irradiation at 300 kHz, the FDP-DD concentration was $163 \pm 5 \text{ ng/ml}$ and the clot lysis was $20.6 \pm 6.5\%$. At 1 MHz, the FDP-DD concentration and clot lysis were $164 \pm 3 \text{ ng/ml}$ and $15.3 \pm 1.1\%$,

Fig. 6. Correlation between the FDP-DD Level Produced from Clots after Incubation in Normal Saline or $2.0 \mu\text{g/ml}$ rt-PA Solution for 1 h at 37°C with Ultrasonic Irradiation and the Decrease in Clot Weight

Correlation coefficient is 0.898 and the regression line is $y = 46.9x - 704$ calculated from 17 clots.

respectively. There were no significant differences produced by ultrasonic irradiation in this case. However, in rt-PA ($2.0 \mu\text{g/ml}$) solution, without ultrasound, the FDP-DD concentration was $957 \pm 129 \text{ ng/ml}$ which was about 6 greater than that measured in normal saline and the clot lysis was $35.6 \pm 2.5\%$. Furthermore, in the rt-PA solution with ultrasonic irradiation at 300 kHz, the FDP-DD concentration was $1669 \pm 290 \text{ ng/ml}$ and the clot lysis was $49.2 \pm 4.5\%$, while at 1 MHz the FDP-DD concentration was $1727 \pm 379 \text{ ng/ml}$ and the clot lysis was $53.4 \pm 6.1\%$. Compared with the group not exposed to ultrasound, the FDP-DD concentration increased by 74% ($p < 0.05$) and the clot lysis by 38% ($p < 0.05$) at 300 kHz. The FDP-DD concentration showed an 80% increase ($p < 0.05$) and the clot lysis a 50% increase ($p < 0.05$) under ultrasound at 1 MHz. The ratio of the increase in FDP-DD concentration was greater than that of the corresponding decrease in clot weight.

Correlation between FDP-DD Concentration and Clot Lysis The FDP-DD concentration and clot lysis were highly correlated ($r = 0.898$, $p < 0.001$).

DISCUSSION

There have been no reports, *in vitro* or *in vivo*, that levels of FDP-DD change in the combined method.

Our *in vitro* determination of FDP-DD revealed that clot lysis of rt-PA with ultrasonic irradiation increased the level of FDP-DD, which is known to accelerate the degradation of fibrin. The following 4 points will be discussed: 1) rt-PA concentration and reaction time, 2) clot lysis by ultrasonic irradiation alone, 3) clot lysis by the combined method, 4) discrepancy between FDP-DD and the degree of clot lysis.

rt-PA Concentration and Reaction Time In the clots produced from 0.8 ml fresh, human blood incubated at

37°C for 4 h, a suitable rt-PA concentration was found to be 2.0 µg/ml and a suitable reaction time was 1 h in our *in vitro* experiment.

This dose was lower and incubation time was longer than in normal clinical practice where intravenous administration of 0.5–1.5 mg/kg rt-PA for 20 min is widely used.^{3,4,7)}

This discrepancy can be explained by the difference between the normal saline solution in our experiment and human blood in a clinical situation.

α₂-Antiplasmin in plasma and plasminogen activator inhibitor secreted from endothelia^{15–17)} in clinical situations reduces the rt-PA clot lysing ability.

Clot Lysis by Ultrasonic Irradiation Alone In our experiment, the clot receiving the ultrasonic irradiation alone showed no significant clot lysis as shown by the FDP-DD levels. Reports have varied regarding the effects of ultrasound on clot lysis. The clot lysis produced by ultrasonic irradiation without any thrombolytic drug was studied by Sobbe *et al.*¹⁸⁾ They were able to destroy clots by ultrasound at 26.8 kHz, with an amplitude of 25 µm in both *in vitro* and *in vivo* experiments. This thrombolytic effect was considered to be the result of micro-cavitation produced by the intense ultrasound.

On the other hand, Furuhashi *et al.*¹⁰⁾ and Lauer *et al.*¹²⁾ reported that the application of ultrasonic irradiation alone to clots did not result in significant lysis. The data from the Furuhashi study was obtained at 0.25 W/cm² and 200 kHz *in vitro* and the Lauer data at 0.375 W/cm² at 1 MHz. The latter group reported that 6–10% lysis was obtained in the rabbit jugular vein thrombosis model. The lack of significant thrombolytic effect from ultrasonic irradiation alone is in accordance with our own data in Fig. 5.

The discrepancies between the Sobbe data and the other data is considered to be due to differences in the ultrasonic frequency and intensity. The frequency characteristics of ultrasonic clot lysis have not yet been studied thoroughly under standardized conditions. We are unable to offer a detailed explanation of these discrepancies in ultrasonic frequency.

The ultrasonic power level, or intensity, is considered to be the most important factor in clot lysis because a high level induces cavitation. In general, cavitation is induced at a power level greater than 1 W/cm² in water at 1013 hPa. Therefore, the destruction of clots observed by Sobbe *et al.*¹⁸⁾ was considered to be caused by cavitation. On the other hand, since the power level used by Furuhashi *et al.*,¹⁰⁾ Lauer *et al.*¹²⁾ and by ourselves (0.4 W, 1 MHz), we below 1 W/cm², it is understandable that no significant clot lysis was seen.

Clot Lysis by the Combined Method Tachibana *et al.*⁹⁾ originated the combined method using thrombolytic drugs with ultrasonic irradiation. They observed qualitatively the usefulness of this method in an *in vivo* study of rat capillaries using urokinase and ultrasound at 23 kHz. In addition, Furuhashi *et al.*¹⁰⁾ experimented on large human clots in an *in vitro* experiment using urokinase and 200 kHz ultrasound, as a model for treating the occlusive thrombi that occur in the coronary artery. They quantified the clot lysis enhancement by measuring the change in clot

weight. On the basis of their data, Kudo¹¹⁾ and Hamano¹⁹⁾ performed *in vivo* experiments using a dog femoral thrombus model. They reported similar results indicating that the combined method reduced the recanalization time to about 40% and the rt-PA dose to about 30%, compared with rt-PA method alone. Lauer *et al.*¹²⁾ also showed that the combined method with rt-PA and intermittent ultrasonic irradiation at 1 MHz increased clot lysis by about 55% in the rabbit jugular vein thrombosis model, compared with rt-PA alone. Simultaneously, they also quantified the enhancement of thrombolysis by measuring the amount of radioisotope-labeled fibrinogen. However, the mechanism of enhancement could not be adequately explained by this experiment because the quantity of radioisotope-labeled fibrinogen did not indicate that degradation of fibrin had taken place. In spite of the fact that thrombolysis enhancement by the combined method has been confirmed by various experiments, it has not been shown clearly whether this phenomenon is due to the destruction of clots which produce the small particles found in fibrin or to the degradation of fibrin to give FDP. This present study shows that the combined method is directly related to the degradation of fibrin because the quantity of FDP-DD is increased. Different ultrasonic frequencies were used in most of the above-mentioned reports so that we could not select the optimum ultrasonic frequency for the combined method. Only in the paper by Lauer *et al.*¹²⁾ it is possible to compare data on the same frequency at 1 MHz. They did not observe any increase in clot lysis by using intermittent ultrasound at an intensity of 0.375 W/cm² *in vitro*; in our case we found an increase in FDP-DD when continuous ultrasound was applied at an intensity of 0.4 W/cm³. This difference can be explained by the geometry of the ultrasonic beam. Lauer *et al.*¹²⁾ located the clot 6 cm from the surface of the transducer (i.d.: 27 mm), while our clot was placed on a glass plate 1 cm from the transducer surface (i.d.: 20 mm). Thus, our data were obtained from clots in the near field of ultrasonic beam. Since the ultrasonic field exhibits a multi-polar distribution, the spatial intensity at polar points is very strong, more than several times greater than the mean power level. Therefore, this may account for the differences between the Lauer study and ours.

Of course, in order to make more quantitative comparisons, it will be necessary to further investigate the relationship involving the beam structure and the distance between the clot and transducer surface.

Discrepancy between FDP-DD and the Degree of Clot Lysis Figure 5 shows that FDP-DD increased by 80% in the combined method using rt-PA and ultrasonic irradiation at 1 MHz and the reduction in clot weight increased by 50%. At 300 kHz, FDP-DD increased by 74% and the reduction in clot weight increased by 38%. From the size of these changes, we can see that FDP-DD measurement is more sensitive for evaluating clots lysis than measuring the decrease in clot weight. However, since there is a high correlation between FDP-DD and the decrease in clot weight, as shown in Fig. 6, the data obtained from clot weight measurements can be used to indicate the degree of clot lysis. It is important to note

that in measuring the degree of clot lysis, clot weight cannot be used instead of FDP-DD measurement because when the decrease in clot weight was 10 or 20%, the FDP-DD level was zero, as shown in Fig. 6. This may be due to the fact that the clot weight measurement includes not only the degradation of fibrin, but also the dissolved clot weight. Evaluation of the actual degradation of fibrin, resulting from the application of rt-PA and enhanced by ultrasonic irradiation, had not been performed quantitatively until we measured FDP-DD from the clot lysis in our experiment.

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