Ultrasound Promotes Penetration of Thrombolytic Agent into Blood Clots and Enhances Thrombolysis


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Abstract

Today, enzymatic thrombolysis employing the fibrin-selective recombinant tissue plasminogen activator (rt-PA) is widely used in the clinical setting for treatment of myocardial infarction, cerebral insult and limb ischemia. However, penetration of rt-PA from the surrounding blood into the thrombus is a very slow process and a rate-limiting step in the reestablishment of blood flow and the maintenance of viability of the affected region, but it can be accelerated when taking place in an ultrasound field (sonothrombolysis). In our research on sonothrombolysis we have studied the effects of therapeutic ultrasound on thrombolysis enhancement in vitro. Clot weight loss measurements and measurements of concentration of the fibrin degradation product D-dimer in the supernatant showed that ultrasound of 2 MHz frequency and 1.2 W/cm² acoustic intensity enforced thrombolysis significantly. By using gel immobilization technique and immuno-histochemistry, we were able to localize plasminogen and rt-PA within the clot. The spatial distributions of these fibrinolytic components showed clearly that ultrasound promoted the penetration of rt-PA into thrombi significantly and broadened the zone of lysis from 9 µm to 21 µm on average. We have shown for the first time that ultrasound facilitates the access of rt-PA to the deeper layers of the clot, thus leading to significantly faster clot dissolution.

1. Introduction

Thrombosis is a partial or entire occlusion of a blood vessel caused by a thrombus (blood clot). Although clots hinder the flow in blood vessels due to their compact structure, they do not represent closed systems and they exchange molecules with their environment. The removing of fibrin deposits is a carefully regulated physiological process, but also very slow because of the very low concentration of human's own thrombolytic agent in the blood. In order to achieve a rapid reestablishment of blood flow, which is necessary to maintain viability of the affected region, clot dissolution must be accelerated pharmacologically by intravenous administration of thrombolytic agent (plasminogen activator (rt-PA) which is a genetically engineered copy of the human's tissue-type plasminogen activator (t-PA) [1]. If the pressure drop across the clot is negligible, the penetration of rt-PA from the surrounding liquid into the thrombus is based solely on diffusion. A narrow lysis front, not exceeding 5-8 µm in depth, moves slowly inwards and fibrin degradation proceeds layer by layer [2].

In addition to enzymatic thrombolysis, the possibility of lysing blood clots by directly applying intravascular ultrasound has long been known. The thrombolytic effect of low frequency ultrasound (20 to 50 kHz) at high intensities (up to 20 W/cm²) is primarily based on mechanical disruption and disintegration of blood clots. In contrast, exposure to higher ultrasound frequencies (1 to 3 MHz) at moderate intensities (up to 4 W/cm²) in conjunction with pharmacologic therapy is not associated with mechanical alterations of the clots, but with enhanced enzymatic activity of the thrombolytic agents. Consequently, numerous studies [3-8] have focussed recently on the investigation of non-invasive ultrasound as a safer and cheaper alternative to catheter based, invasive ultrasound, also requiring less technical and logistical factors for clinical implementation.

It has been assumed that ultrasound exerts its beneficial effects on thrombolysis by promoting the transport of enzymes into clots and by increasing the uptake of rt-PA [9]. However, to date – at least to our knowledge – no data are available on the distribution and the localization of fibrinolytic enzymes in blood clots during ultrasound treatment.

Therefore, in this study we tested the hypothesis whether ultrasound changes the distribution and localization of fibrinolytic enzymes within the blood clot and on the clot surface and thus supports enzymatic thrombolysis by rt-PA.

2. Materials and methods

2.1. Sonication set up

A tubing containing a fresh blood clot and the thrombolytic solution (0.9% degassed saline solution with 3000 U/mL rt-PA) was placed in a sonication chamber at a distance of 3 cm from the transducer (Fig. 1). The chamber was filled with temperature-controlled (36°C) degassed water. The tubing was made...
of silicone copolymere with an inner diameter of 4.8 mm and 0.8 mm wall thickness. The acoustic properties of this material are similar to those of human blood vessels [10].

![Figure 1](Image)

**Figure 1** Sonication set up

The composite transducer, with its fundamental resonance frequency at 1.95 MHz, consisted of two PZT (lead-zirconate-titanate) piezoceramics (25x25x1 mm³) electrically connected in series and bonded side by side on a glass carrier. The transducer was driven by a highly specialized power frequency synthesizer. In order to obtain a propagating wave field, a specially designed absorber made of paraffin was placed opposite to the transducer.

Ultrasound was applied intermittently with 1 Hz pulse repetition frequency and with 1:1 ratio for sonication versus rest time (50% duty cycle). The rationale for applying the intermittent ultrasound is the shorter effective exposure duration of the clots, which would be of advantage in ultrasound application in vivo. The energy deposited within the tissue, which may be converted into heat during intermittent sonication, is half the energy that would be deposited in the tissue during continuous sonication.

The average acoustic intensity measured by a radiation balance at the locus of the thrombus was 1.2 W/cm².

The temperature rise within the chamber (at the thrombus location) as measured with a Pt-100 thermometer, did not exceed 1°C during 60 min of sonication.

2.2. Evaluation of thrombolysis

Fresh blood clots were formed as described elsewhere [11]. Each clot was weighed on an analytic balance before treatment. Initial clot weight was (450 ± 50) mg. After treatment, the partially dissolved clot and the solution were carefully removed from the tubing and clot weight was measured again. The lysing effect was determined as the percentage decrease in clot weight after treatment. The collected solution containing cleavage products was analysed for the fibrin degradation product D-dimer by using a specific ELISA kit.

2.3. Clot immobilization and immuno-histochemistry

In the applied model of thrombolysis, there was no pressure gradient across the clot, and the rt-PA induced fibrinolysis took place on the surface of the clot. Therefore, it was of essential importance for our study to develop a method of clot immobilization, which would preserve the clot surface from damaging. For this purpose, we adapted the polymer gelation technique developed by Gherardini et al. [12], to protect the thrombi from damage due to manipulation and to facilitate their sectioning in the cryostat.

Gel solution was prepared by adding Agar Agar (10 g/L) in saline solution. At this concentration, the gel was liquid at 37°C and started to harden fast and evenly after decreasing the temperature below 30°C. If necessary for the experiment, rt-PA was added to a final concentration of 3000 U/ml. After treatment, the tubing was immediately placed into an ice-water bath (4°C) to initiate solidification of the gel. The formed gel containing the clot was carefully removed from the tubing, placed in an embedding container, fully covered with O.C.T. compound, snap frozen in liquid nitrogen and stored at -80°C until cutting. The samples were cut into 4 µm thick slices along the direction of ultrasound propagation by using a cryostat chamber. The immuno-histochemical labeling of plasminogen and rt-PA was performed on cryostat sections of the thrombi. Primary antibodies used were monoclonal antibodies (mouse immunoglobulin G, IgG) against human plasminogen and polyclonal antibodies (rabbit IgG) against human t-PA. Slides were incubated in a wet chamber for 30 minutes with secondary fluorescent antibodies under light protection. As secondary antibodies we used fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG.

Digital imaging fluorescence microscopy was used for analysis. The obtained images show the spatial distributions of plasminogen and t-PA within the clots at the end of the treatment time.

3. Results

Two types of experiments were performed. The objective of the first set of experiments was to investigate quantitatively the enhancing effect of ultrasound on thrombolysis (Fig. 2). The objective of the second set of experiments was to study the distribution of fibrinolytic components within clots exposed to ultrasound (Fig. 3 and Fig. 4).

3.1. Enhancing effect of ultrasound on thrombolysis

Only a small weight loss was observed in untreated clots (11.0 ± 3.8 %). Exposure of clots to ultrasound in the absence of rt-PA resulted in a pronounced clot dissolution (25.3 ± 3.9) %, comparable to that of the group treated with rt-PA only (19.9 ± 4.3) %.
clots were exposed to ultrasound in the presence of rt-PA, a further weight reduction was seen (35.2 ± 6.9) %.
Similar results were obtained when FDP-DD levels in the supernatants of clots after the respective treatment were measured (Fig. 2).

A significant colocalization of t-PA and plasminogen on the clot surface was observed in all experimental groups.

3.2. Immuno-histochemical analysis of blood clots

Typical fluorescence microscopy findings for the clots treated with ultrasound alone, rt-PA alone and rt-PA in combination with ultrasound, respectively, are presented in Fig. 3. The images of rhodamine-labeled rt-PA distributions and FITC-labeled plasminogen distributions were taken separately and then merged together to identify overlapping regions. The image of control is not shown, because there was no signal detected. The measurements of the corresponding fluorescence widths are shown in Fig. 4.

When the clots were exposed to ultrasound in saline solution only (Fig. 3a), both t-PA and plasminogen were present as narrow zones at the clot surface as revealed by thin layers of red and green, respectively, fluorescence staining. However, the layer of plasminogen staining (9.8 ± 2.1) µm was eightfold deeper than the accumulation of t-PA (1.6 ± 1.1) µm. Due to the fact that neither rt-PA nor plasminogen had been added to the surrounding solution, the detected t-PA and plasminogen must represent intrinsic enzymes, which had moved from the inside of the clot to its surface, probably due to the squeezing effect of the acoustic radiation pressure. When rt-PA was present in the solution surrounding the clot in the absence of ultrasound (Fig. 3b), both rt-PA and plasminogen accumulated within a thin superficial layer of the clot (t-PA: 8.9 µm ± 2.6 µm; plasminogen: 14.1 µm ± 5.6 µm). The application of ultrasound in addition to rt-PA broadened the zones of red and green fluorescence staining significantly (p<0.0001) indicating the deeper penetration of rt-PA into the clot and the migration of plasminogen from the inside of the clot to its surface (Fig. 3c). The depths of t-PA and plasminogen fluorescence increased to (21.2 ± 7.2) µm and to (41.1 ± 10.8) µm, respectively.

Figure 2. Clot weight loss of thrombi (green) and concentration of fibrin degradation product Ddimer in supernatant (blue). Each group contained 10 clots. Treatment time was 60 min. Results are given in (mean±SD).

Figure 3. Spatial distribution of plasminogen (green) and t-PA (red) in blood clots exposed to (a) ultrasound only, (b) rt-PA only, and (c) rt-PA in combination with ultrasound. The zones of overlapping appear yellow after merging of the respective images.

Figure 4. Width of fluorescence for different types of clot treatment. Each group contained 4 clots. One surface segment per clot slice quadrant was taken for examination. Fluorescence width was measured along five different lines, chosen at random within each segment, amounting to 80 measurements per experimental group.
4. Discussion

To visualize the spatial distribution of fibrinolytic components within blood clots during sonothrombolysis, we have established a reliable and versatile protocol for clot immobilization within a thermogel-matrix, which protects the clot surface from damaging due to manipulation and facilitates the further processing of the frozen sections. By using the immunohistochemical method of double staining, we were able to localize both rt-PA and plasminogen within the same sections. We found that combined exposure of blood clots to rt-PA and ultrasound resulted in a significantly deeper penetration of rt-PA into the clot when compared to clots treated with either rt-PA or ultrasound alone. This significantly broader zone of lysis correlates well with a significantly enhanced clot weight reduction and increased FDP-DD levels, when compared to clots treated either with rt-PA or ultrasound alone. Contrary to many other published works, the application of ultrasound alone in our experiments resulted in a significant clot lysis, even exceeding the one achieved with rt-PA alone. Not only the clot weight measurements and FDP-DD analysis, but also the immunohistochemical analysis support this finding. Whereas plasminogen and intrinsic tissue plasminogen activator (t-PA) could not even be detected in clots incubated in saline, their concentrations on the surfaces of clots treated with ultrasound without rt-PA increased significantly. There was only one pool of t-PA and plasminogen inside a clot and no rt-PA and plasminogen in the outer medium, which means that the proteins must have moved from the inside to the surface of the clot probably due to the action of acoustic radiation pressure. Therefore, ultrasound facilitated not only the transport of rt-PA from the surface to the inner parts of the thrombus, but also the transport of fibrinolytic components from the inner parts to the surface.

Based on these facts we conclude that ultrasound-induced changes in the spatial distribution of fibrinolytic enzymes in whole blood clots are responsible for the augmenting effects of ultrasound on enzymatic thrombolysis. Thus, our findings describe a new aspect of the fibrinolytic potential of high frequency ultrasound.

5. Acknowledgment

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6. References


