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Fibrin degradation during sonothrombolysis – Effect of ultrasound, microbubbles and tissue plasminogen activator

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ABSTRACT

Microbubbles (MB) have previously demonstrated their great potential to enhance sonothrombolysis (STL) in vascular occlusions. Nonetheless the impact of MB-mediated STL on the fibrin network of clots has not been investigated up to now. The aim of this study was to evaluate the ability of combined ultrasound (US) and MB to degrade the fibrin in the presence or absence of a thrombolytic drug. Human blood clots containing radiolabeled-fibrin were exposed to different combinations of recombinant tissue plasminogen activator (rtPA), US (1.0 MHz; 1.3 MPa; 0.08% duty cycle) and MB. Three different techniques were used for the assessment of clot lysis: diameter loss, release of radioactive-fibrin degradation products (FDP) and D-Dimer assay. On one hand, the combination US + MB without rtPA induced a diameter loss (0.18 ± 0.01 mm), but no fibrin degradation, as revealed by the absence of radioactive-FDP release (0.5 ± 0.1%). On the other hand, the combination rtPA + US + MB enhanced both the diameter loss (0.77 ± 0.07 mm) and fibrin degradation (50.3 ± 2.4%) compared to rtPA alone (0.24 ± 0.01 mm and 36.5 ± 1.7%; $p < 0.001$), thus demonstrating a strong synergistic effect. The D-Dimer assay further confirmed the radioactivity results. Sonothrombolysis with MB greatly improves rtPA-mediated clot lysis and fibrin degradation was shown to be a key factor of the process. Studies addressing the clinical impact of this technique are warranted.

1. Introduction

Among new therapeutic options for ischemic stroke treatment, sonothrombolysis (STL) appears as one of the most promising. Initially based on the use of ultrasound (US) waves only, STL has taken on a new dimension with the introduction of microbubbles (MB) to reinforce the US action [1]. Since then, several studies have illustrated the great potential of MB to improve STL [2–9].

Microbubbles enhance US-induced bioeffects by providing cavitation nuclei to the medium, thus decreasing considerably the acoustic cavitation threshold. Although the exact mechanisms involved in the STL process with MB remain partially unraveled, cavitation-related phenomena play an essential role [4,10,11]. Indeed, cavitation is able to induce a mechanical stress at the liquid-clot interface by generating microstreaming and violent microjets. Therefore cavitation could, on one hand, cause direct mechanical damage to the clot, resulting in better accessibility of thrombolytic drug to the fibrin network [9,12]. On the other hand, it could promote transport of the drug and enhance its diffusion inside the clot, thus increasing the likelihood of drug-fibrin interactions [2].

The clot stability is ensured by a three dimensional network of fibrin [13,14]. Degradation of this network is a key element in the clot destruction process and in the risk of re-occlusion. Up to now, the impact of US combined with MB on the fibrin network integrity has not been evaluated. In fact most studies have used either clot mass loss or diameter loss to quantify the clot lysis without considering the fibrin.

The aim of the present work was to evaluate whether 1 MHz-US and a new MB formulation (BR38) could induce fibrin degradation *in vitro*, with and without a thrombolytic drug. For that purpose, human blood clot containing radiolabeled-fibrin, were prepared.

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The clots were exposed to different treatment conditions and the lysis assessed by three quantitative techniques: clot diameter loss, release of radioactive-fibrin degradation products (FDP) and D-Dimer assay. The latter two were used more specifically to assess the fibrin degradation of the clot.

2. Materials and methods

2.1. Materials

Trizma® hydrochloride buffer solution, sodium chloride, sodium citrate tribasic dihydrate, bovine serum albumin (BSA), calcium chloride dihydrate and 6-aminocaproic acid (6AA) were purchased from Sigma–Aldrich (Buchs, Switzerland). Human fibrinogen, plasminogen-depleted (lyophilized in 20 mM sodium citrate-HCl, pH 7.4) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany), reconstituted according to the supplier at 44.25 mg/mL with water, and then stored at −80 °C until use. Iodine-125 (^{125}I) radionuclide was purchased from Perkin Elmer, Inc. (Waltham, MA, USA). Frozen citrated human plasma was purchased from Milan Analytica AG (Magden, Switzerland), defrosted at 37 °C, then filtered through 0.22 μm Steripak GP20 filter (Millipore, Billerica, MA, USA) aliquoted and then stored at −20 °C. Plasma was defrosted at 37 °C before use in STL experiments. The recombinant tissue plasminogen activator (rtPA) was Actilyse® (Boehringer Ingelheim, Basel, Switzerland). It was reconstituted according to the supplier's indications at 1 mg/mL and stored at −80 °C [15,16].

2.2. Methods

2.2.1. Radioiodination of human fibrinogen

Human fibrinogen was radiolabeled with ^{125}I using the iodogen technique [17]. Briefly, 100 μL of fibrinogen (4.4 mg/mL) in 0.125 M Tris.HCl, 0.15 M NaCl, 20 mM citrate, pH 6.8 and 7.4 MBq (200 μCi) of ^{125}I were mixed into a Pierce® iodination tube (Thermo Scientific, Rockfold, IL, USA) and allowed to react for 15 min at room temperature. The solution was then removed from the tube and diluted with 0.5 mL of 25 mM Tris.HCl, 0.4 M NaCl, 20 mM citrate, pH 7.5. Labeled fibrinogen was purified using a 10 mL polyacrylamide 6000 desalting column (Thermo Scientific, Rockford, IL, USA) after saturation with BSA and equilibration with 25 mM Tris.HCl, 0.4 M NaCl, 20 mM citrate, pH 7.5. The labeling efficiency was 86%. The concentration of the final ^{125}I-fibrinogen solution was 0.3 mg/mL, as determined by UV spectrophotometry (Lambda 35 UV/VIS spectrometer, Perkin Elmer Inc., Waltham, MA, USA). The specific activity of the labeled fibrinogen was approximately 14.8 MBq/mg.

2.2.2. Preparation of human blood clots

Radioactive (^{125}I-clots) and non-radioactive clots were prepared following the same protocol. They were used for radioactive-fibrin degradation products (^{125}I-FDP) release experiments and D-Dimer assays, respectively. The protocol was adapted from a previous study [15].

Fresh venous human blood was drawn (Neutral S-Monovette®, Sarstedt, Nümbrecht, Germany) from healthy volunteers after signature of an informed consent. Rapidly, 200 μL of either ^{125}I-fibrinogen or non-labeled fibrinogen solution (0.3 mg/mL in 25 mM Tris.HCl, 0.4 M NaCl, 20 mM citrate, pH 7.5), and 20 μL of 0.5 M $CaCl_2$ were mixed to 9 mL of blood. Calcium was added to annihilate the effect of citrate present in the fibrinogen solution. Four milliliters of blood were then transferred into glass test tubes (Pyrex®, 16 × 100 mm) containing five glass tubes of 3.4 mm i.d., through which a silk suture thread (Silkam® black USP 4/0, B. Braun Aesculap A.G., Tuttlingen, Germany) had been previously stretched. After 6-h incubation at 37 °C, the clots were removed from the glass tubes and stored in phosphate-buffered saline at 4 °C for 1–3 days before use.

No macroscopic differences were observed between radioactive and non-radioactive clots. They had a cylindrical shape with an initial mean diameter and length of 2.15 ± 0.08 mm and 25 ± 2 mm, respectively (n = 74).

To evaluate the incorporation of ^{125}I-fibrinogen in the radioactive-clots (clottability), 100 μL of radioactive blood and 100 μL of serum (containing the unincorporated ^{125}I-fibrinogen) were sampled, respectively before and after clot formation, and the radioactivity was counted (Cobra™ II Auto-Gamma®, Packard Instrument Company, Meriden, CT, USA). Clottability was calculated as follows:

$$clottability\ (\%) = \frac{blood\ radioactivity - serum\ radioactivity}{blood\ radioactivity} \times 100$$

2.2.3. In vitro sonothrombolysis

The experimental setup for STL experiments was described in a previous study [15]. It was used in this study with minor modifications (Fig. 1).

Briefly, clots were held in a transparent PVC tube of 3.2 mm i.d. in a 37 °C thermostated water bath. Human plasma was circulated through the tube at a flow rate of 21 mL/min, corresponding to an approximate flow velocity of 80 mm/s along the clot. This flow velocity was chosen to be in the range of the ones observed in partially occluded middle cerebral artery [18]. The clots were exposed, during 60 min, to different combinations of rtPA, US and MB. The rtPA was added directly to the human plasma at a concentration of 3 μg/mL, at the beginning of the experiment. This concentration corresponds to typical plasma concentrations at steady state after intravenous administration to patients [19].

Instead of a focused transducer as used in our previous study [15], an unfocused, custom-made 1.0 MHz single element transducer (working distance 30 mm; Philips Research, Briarcliff Manor, NY, USA) was chosen here to insonify the clots. This allowed a larger area of the clots to be exposed to the US beam. The transducer was driven by an arbitrary-waveform generator (Model 8024, Tabor Electronics Ltd., Tel Hanan, Israel), with a voltage divider (470 Ω series-resistor) connected to a 150-W power amplifier (A150, Electronics & Innovation, Rochester, NY, USA). Ultrasound was applied as 1-ms long sinusoidal tone bursts and stopped for 1249 ms (duty cycle of 0.08%) to allow complete MB replenishment of the tube containing the clot between successive bursts. A peak-negative pressure (PNP) of 1.3 MPa inside the tube was applied. This PNP was calculated from measurements performed in free field and corrected for the tube-wall attenuation of 2 dB at 1.0 MHz, as previously described [15]. An acoustic absorber (Aptflex F28, Precision Acoustics Ltd., Dorchester, UK) was placed behind the tube containing the clot to minimize US reflection.

BR38 phospholipid microbubbles [20] (Bracco Suisse S.A., Geneva, Switzerland), reconstituted with 5 mL of 0.9% NaCl, were infused in the plasma at 4 × 10^5 MB/min using a VueJect® pump (Bracco Suisse S.A., Geneva, Switzerland). BR38 microbubbles are formulated with neutral phospholipids, distearoylphosphatidylcholine and a pegylated phospholipid (DPPE-MPEG5000), and palmitic acid [20]. The entrapped gas is a mixture of perfluorobutane and nitrogen gases (35/65 vol/vol) [20]. The mean diameter in number and the median diameter in volume were, 1.47 ± 0.02 μm and 3.24 ± 0.07 μm (mean ± SD), respectively [20].