Clot penetration and fibrin binding of amediplase, a chimeric plasminogen activator (K_2tu-PA)

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Summary

Amediplase (K_2tu-PA) is a hybrid plasminogen activator, consisting of the kringle 2 domain of alteplase and the protease domain of urokinase. The objective of this study was to determine the *in vitro* clot penetration of amediplase in relation to its fibrin binding and to compare the properties with those of alteplase.

The clot lysis activity of amediplase in internal clot lysis models (both purified system and plasma system) was about 10 times less than that of alteplase. The clot lysis activity of amediplase in an external clot lysis model (plasma system) was similar to that of alteplase at therapeutic concentrations around 1 µg/ml. The fibrin-clot binding properties of amediplase and alteplase were studied in a purified system as well as in a plasma system. In both systems amediplase bound to fibrin although to a significantly lower extent than alteplase. The binding of amediplase or alteplase did not increase during plasmin-mediated degradation of fibrin. The binding of amediplase was fully inhibited by epsilon-aminocaproic acid, indicating that the observed binding was specific and occurred via the lysine binding site in the kringle of amediplase. Clot penetration was studied during pressure-driven fluid permeation using syringes containing plasma clots. Amediplase was able to enter the clot without significant hindrance, while alteplase was concentrated on the top of the plasma clot and hardly entered into the inner parts of the clot. Diffusion-driven clot penetration was studied during clot lysis using confocal microscopy. Alteplase was detected on or close to the clot surface, while two-chain urokinase, which has no affinity to fibrin, was also detected deep inside the clot. Amediplase showed a penetration behaviour, which was distinct from that of alteplase and similar to that of two-chain urokinase.

We concluded that the fibrin binding of amediplase is moderate and does not hinder clot penetration under permeation-driven or diffusion-driven transport conditions. Enhanced clot penetration, especially in large clots, could allow a more efficient lysis during thrombolytic therapy.

Keywords

Amediplase, alteplase, fibrin binding, clot penetration, thrombolysis

Thromb Haemost 2004; 91: 52–60

Introduction

Amediplase (K_2tu-PA) is a single-chain hybrid plasminogen activator, comprising amino acids 1-3 and 176-275 (the kringle 2 domain) of tissue-type plasminogen activator (t-PA or alteplase) and amino acids 159-411 (the protease domain) of single-chain urokinase-type plasminogen activator (scu-PA) (1). T-PA has a strong affinity to fibrin (2), which is ascribed to the finger domain and to a lesser extent to kringle 2 domain of t-PA (3,4). Scu-PA has no affinity to fibrin. Due to the presence of kringle

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Received July 3, 2003
Accepted after revision September 8, 2003

Financial support:
This study was financially supported by Menarini Ricerche S.p.A., Rome, Italy

Prepublished online November 7, 2003 DOI: 10.1160/TH03-07-0435

2, K_2tu-PA has a moderate affinity to forming fibrin (5). The effect of the presence of kringle 2 on the fibrinolytic properties of K_2tu-PA has been extensively described by Colucci et al (5). Animal studies in dogs (coronary thrombosis model) and rabbits (jugular vein thrombosis model and ear bleeding model) indicated that K_2tu-PA has potent thrombolytic properties with a prolonged plasma half-live, which may allow administration via an intravenous bolus injection (6-8). After a dose finding study in patients with acute myocardial infarction (9), a phase II trial was performed in 241 patients with acute myocardial infarction, which showed a high TIMI grade 3 flow (58%) and TIMI grade 2 and 3 flow (76%) at 60 min (10). Phase III clinical trials in patients with myocardial infarction are currently in preparation (11).

Clot penetration of plasminogen activators during thrombolytic therapy may be an important determinant of the regulation of the speed of the lytic process (12). Penetration of plasminogen activators occurs by two different mechanisms (13, 14). The first one is based on diffusion, which is an efficient mechanism of transport over a short distance. The second one is based on pressure-driven permeation of fluid containing the plasminogen activators through the clot. Both mechanisms may play a role in a way that depends on the type and the position of the clot (venous versus arterial, retracted versus non-retracted, occluding versus non-occluding, etc). Within a single clot the architecture usually varies. As a result lysis of some parts may depend on permeation while lysis of other parts may depend on diffusion.

Clot penetration of plasminogen activators is probably hampered by binding to the fibrin network of the clot (15, 16). We hypothesized that clot penetration of amediplase is better than that of alteplase, because amediplase has a lower affinity to fibrin than alteplase. The objective of this study is to determine the *in vitro* clot penetration of amediplase in relation to its fibrin-binding properties.

Materials and methods

Materials

Amediplase (code-name MEN 9036, lot 02/99, vials of 30 mg amediplase containing 10.62 mg succinic acid and 300 mg mannitol) was supplied by Menarini Biotech. Each vial was dissolved in 6 ml of water and stored in aliquots of 0.5 ml at –70°C. Alteplase (t-PA) was from Boehringer Ingelheim and Ukidan (two-chain urinary urokinase, specific activity 170 IU/µg) from Serono. Bovine thrombin was from Organon Teknika, bovine albumin was from Sigma (BSA A7030), aprotinin (Trasylol) from Bayer and heparin from Leo Pharmaceuticals.

Internal clot lysis system

Aliquots of 500 µl cold human citrated plasma were supplemented with 1 µl of increasing concentrations of amediplase or alteplase, vigorously stirred, clotted with 7 µl of a calcium chloride (20 mM, final concentration) and bovine thrombin (1 NIH unit/ml, final concentration) mixture, and finally incubated at 37°C. The disappearance of all air bubbles from the lysing clot indicated the lysis time. Clot lysis in a purified system was performed by replacing citrated plasma by 3.2 mg/ml human plasminogen-containing fibrinogen (Chromogenix) diluted in clot lysis buffer (CLB) consisting of 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 10 mg/ml BSA.

External plasma clot lysis system

Aliquots of 36.5 µl human citrated plasma were supplemented with 2.7 µl FITC-labelled fibrinogen (plasminogen, von Willebrand factor and fibronectin depleted human fibrinogen from Enzyme Research Laboratories, 0.3 mg/ml final concentration), and clotted with 0.8 µl of a calcium chloride (20 mM, final concentration) and thrombin (1 NIH unit/ml, final concentration) mixture in plastic 4-ml tubes of Greiner (No 112101). After an incubation period of 3 min at 37°C the clots were covered with 240 µl citrated plasma supplemented with increasing concentrations of amediplase or alteplase and the incubation was continued at 37°C on a slow shaking table for 240 min. At intervals of 30 min 5 µl samples were taken from the plasma, diluted with 495 µl buffer and the released FITC-labelled fibrin degradation products were determined in a spectrofluorometer.

Purified fibrin clot binding of amediplase and alteplase

One hundred µl fibrin clots were prepared by mixing human fibrinogen (Chromogenix, 3.2 mg/ml, final concentration), amediplase (160 ng/ml, final concentration) or alteplase (15 ng/ml, final concentration), calcium chloride (20 mM, final concentration) and bovine thrombin (1 NIH unit/ml, final concentration) in clot lysis buffer (CLB). Fibrin clots that were not allowed to lyse contained, in addition, 1000 KIU/ml Trasylol. After incubation of the fibrin clots for 10-15 min at 37° C, the clots were immediately cooled down in ice water, detached from the tube walls with a small spatula, and centrifuged for 2 min at 13,000 rpm. The supernatants were diluted in cold dilution buffer of the ELISA (see below), to which Trasylol (100 KIU/ml) was added, and were then analysed in the ELISA. The amounts determined in the ELISA were subtracted from the amounts found in control incubations that were not clotted with calcium and thrombin, and the differences indicated the amounts of plasminogen activator bound to the fibrin clots.

Plasma clot binding of amediplase and alteplase

One hundred µl plasma clots were prepared by mixing human citrated plasma (88%, final concentration), amediplase (1400 ng/ml, final concentration) or alteplase (70 ng/ml, final concentration, calcium chloride (20 mM, final concentration)

and bovine thrombin (1 NIH unit/ml, final concentration). Plasma clots that were not allowed to lyse contained, in addition, 1000 KIU/ml Trasylol. The incubation of the clots and the determination of the extent of plasminogen activator binding were carried out as described above for the purified fibrin clots.

Enzyme immunoassays of alteplase and amediplase

Alteplase was measured with the Imulyse ELISA kit of Biopool. Alteplase and its two-chain form as well as complexes with plasma proteinase inhibitors are equally well detected in this assay. Amediplase was measured by combining the catching antibody of a home-made ELISA for urokinase (TNO Prevention and Health, Leiden, NL) with the tagging antibody of the Imulyse kit for alteplase (Biopool). Two-chain amediplase (prepared by treating amediplase with plasmin) yielded a signal that was about 10% higher than that of amediplase. However, two-chain amediplase lost a significant part of its reactivity in the ELISA upon incubation in plasma, most likely due to complex formation with plasma proteinase inhibitors. This meant that fibrin binding experiments of amediplase in a plasma system in which plasmin is generated, could not be performed.

Clot penetration by fluid permeation using intact plasma clots

Citrated platelet-poor pooled plasma from healthy donors was recalcified at room temperature by adding $CaCl_2$ to a final concentration of 25 mM. A portion of 350 µl was immediately pipetted into a 1 ml plastic luer tuberculin syringe (Codan Medical ApS, Rødby, Denmark) which was closed at the bottom. A clot (0.166 cm^2 x 2.1 cm) was formed, incubated overnight at room temperature and stored for 1 to maximally 3 days at 4°C. The syringe was connected to a pump (Gilson Minipulse 3) and an amediplase or alteplase solution was drawn into the clot for one hour at room temperature at a speed of 200 µl/hour. The plasminogen activator solution consisted of 28 nM amediplase (1.2 µg/ml) or 28 nM alteplase (2.0 µg/ml) in pooled citrated plasma containing 100 KIU/ml aprotinin and 3 U/ml heparin. With this procedure the upper surface of the clot remained stable and no channels were formed during the permeation.

After the experiment the syringe was disconnected and snap-frozen in pieces of dry-ice and stored at –20°C. In this manner the clot surface was minimally distorted. To determine the spatial distribution of amediplase and alteplase, the bottom of the syringe as well as the upper part of the syringe, just above the clot surface, was rapidly (to prevent thawing) removed by cutting with a dental sawing machine (Faro, Italy). The remaining part of the syringe containing the clot was embedded in Tissue-Tek, mounted and serially sliced every 60 µm using a cryotome (Microm, Heidelberg, Germany). Every ten consecutive slices (about 10 µl) were pooled in Eppendorf tubes and extracted with 230 µl phosphate-buffered saline supplemented with 2 M KSCN, 1 mg/ml bovine serum albumin and 0.01% Tween 80. The tubes were shaken for 30 min at room temperature, centrifuged and the extracts were collected and assayed in the ELISAs for amediplase and alteplase (see above). The starting solutions of amediplase and alteplase were used for calibration of the ELISA.

Clot penetration by diffusion during clot lysis

The clot penetration of various plasminogen activators during plasma clot lysis was studied by assessing increased FITC-labelled plasminogen binding to fibrin fibres in those areas where fibrin degradation took place as a result of the presence of a plasminogen activator. The experimental system for visual-

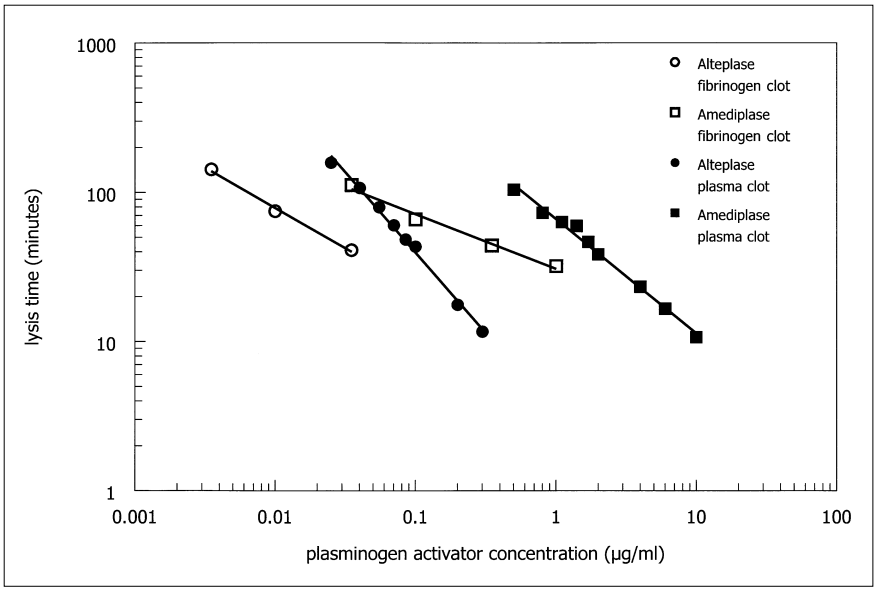

Figure 1: The activities of amediplase and alteplase in an internal clot lysis system with purified fibrin clots or plasma clots. Increasing concentrations of the two plasminogen activators were incorporated into the clots and the lysis times were determined with the air bubble method.

isation of increased FITC-plasminogen binding to fibrin fibres has been described before (15). In brief, citrated platelet-poor plasma containing 0.3 µM FITC-plasminogen was clotted with thrombin (final concentration 1.4 NIH U/ml) between two parallel glass slides separated by a spacer about 0.2 mm high. After a few minutes a clot with an approximate diameter of 2-3 mm was formed firmly attached to the parallel glass slides, and then the remaining volume of the chamber was filled with plasma containing 0.3 µM FITC-plasminogen and 1 µg/ml (24 nM) amediplase, or 1.9 µg/ml (27 nM) alteplase or 300 U/ml (35 nM) two-chain urokinase-type plasminogen activator. The clot was incubated at room temperature for about 50 min and images were taken during ongoing lysis using a confocal fluorescence microscope, as described before (15).

Results

Clot lysis activities of amediplase and alteplase

In order to characterise the two plasminogen activator preparations, amediplase and alteplase were tested in a variety of clot lysis assays, which are based on internal clot lysis (plasminogen activators incorporated into the clot by adding them to the mixture before clotting) as well as on external clot lysis (plasminogen activators applied from the outside to the clot after clot formation).

Figure 1 shows the results of internal clot lysis, both with plasma clots and with purified fibrin clots. The clot lysis times decreased at increasing plasminogen activator concentrations. Using plasma clots about 10-30 times (on a weight basis) more amediplase was required than alteplase to obtain the same clot lysis time. Using purified fibrin clots lower plasminogen activator concentrations were required than with plasma clots, because of the absence of plasma protease inhibitors. Again, about 5-15 times (on a weight basis) more amediplase was required than alteplase to obtain the same clot lysis time.

Figure 2 shows the time-dependent results of external plasma clot lysis in which the plasminogen activators were added to the plasma above the preformed clots. Both amediplase (Fig. 2A) and alteplase (Fig. 2B) showed sigmoidal-type lysis curves, but the characteristics of these curves differed for amediplase and alteplase. The 50% lysis times were longer for amediplase than for alteplase at low plasminogen activator concentrations (Fig. 2C), but they became similar at higher concentrations (around 1 µg/ml). Likewise, the maximal slopes of the lysis curves of Figures 2A and 2B (i.e. maximal speed of lysis) were lower for amediplase than for alteplase at low plasminogen activator concentrations, but they became similar or even higher for amediplase at concentrations around or above 1 µg/ml (not shown). It is interesting to note that both the 50% lysis time and the slope were similar for the two activators at therapeutic concentrations.

Fibrin-binding properties of amediplase and alteplase

The fibrin-binding properties of amediplase and alteplase were studied in a purified system or plasma system by adding the plasminogen activators to plasminogen-containing fibrinogen

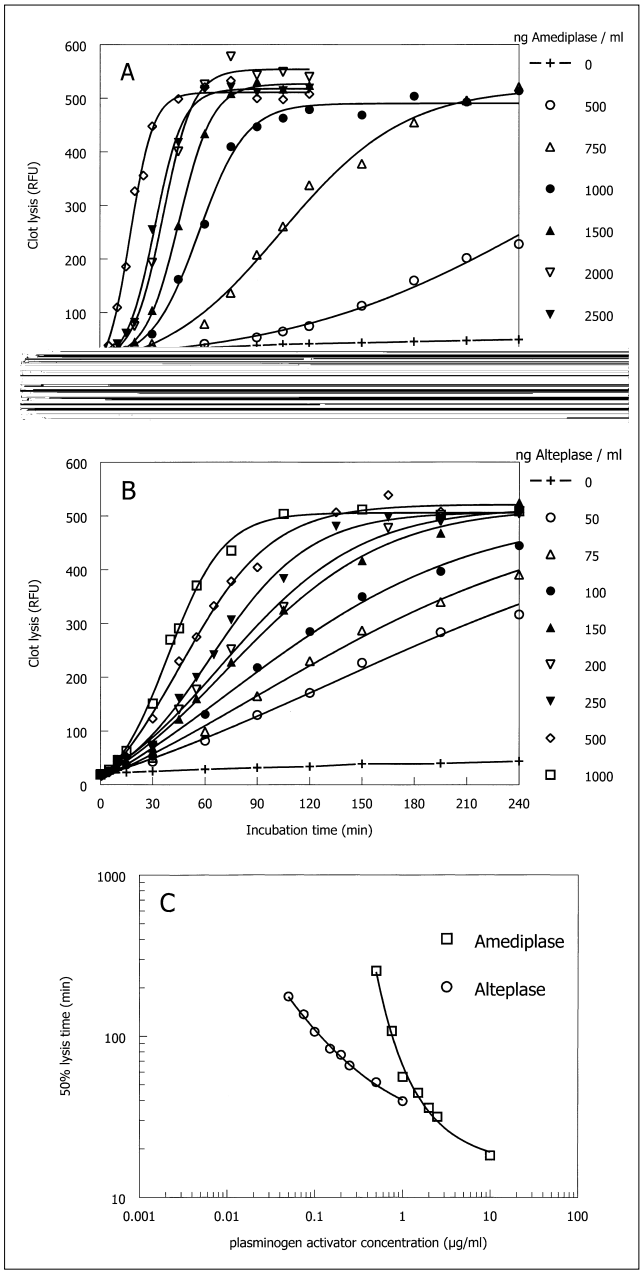

Figure 2: The activity of amediplase (A) or alteplase (B) in an external plasma clot lysis system. Preformed plasma clots labelled with FITC-fibrin were covered with plasma containing increasing amounts of amediplase or alteplase and the time-dependent release of FITC-labelled fibrin degradation products into the plasma was determined with a fluorometer and expressed in relative fluorescence units (RFU). Figure C compares the fifty per cent lysis times of amediplase and alteplase derived from the curves shown in Figures A and B.

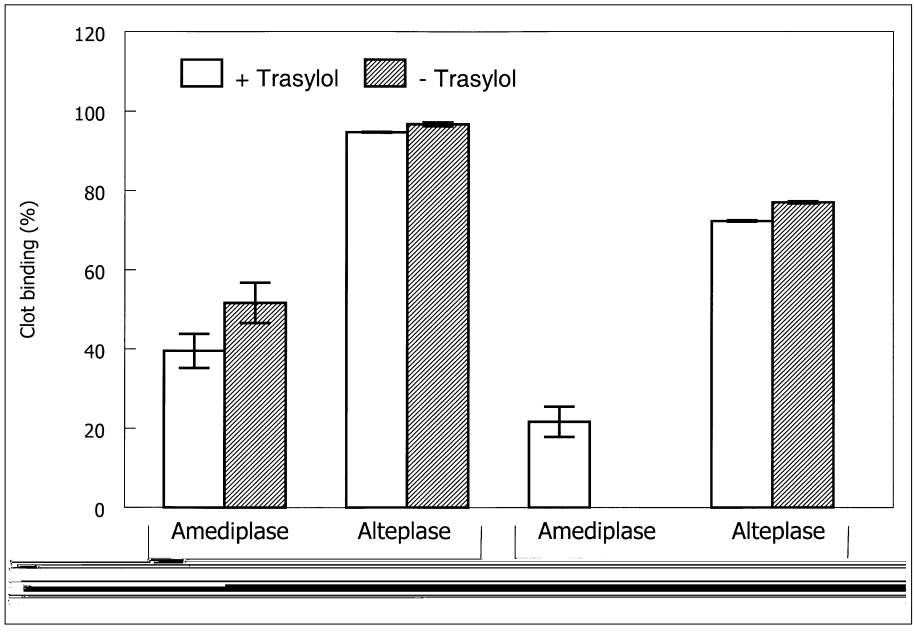

Figure 3: Binding of amediplase and alteplase to purified fibrin clots (3.2 mg/ml) or plasma clots, both in the presence and in the absence of 1000 KIU/ml Trasylol. The incubation time was 10-15 min. The amounts of amediplase and alteplase used in the binding experiments (see Materials and Methods for details) would lyse the clots in the absence of Trasylol in 60 min. The binding of amediplase to plasma clots in the absence of Trasylol could not be quantified (see Marterials and Methods). The error bars indicate the variation in duplicate binding experiments.

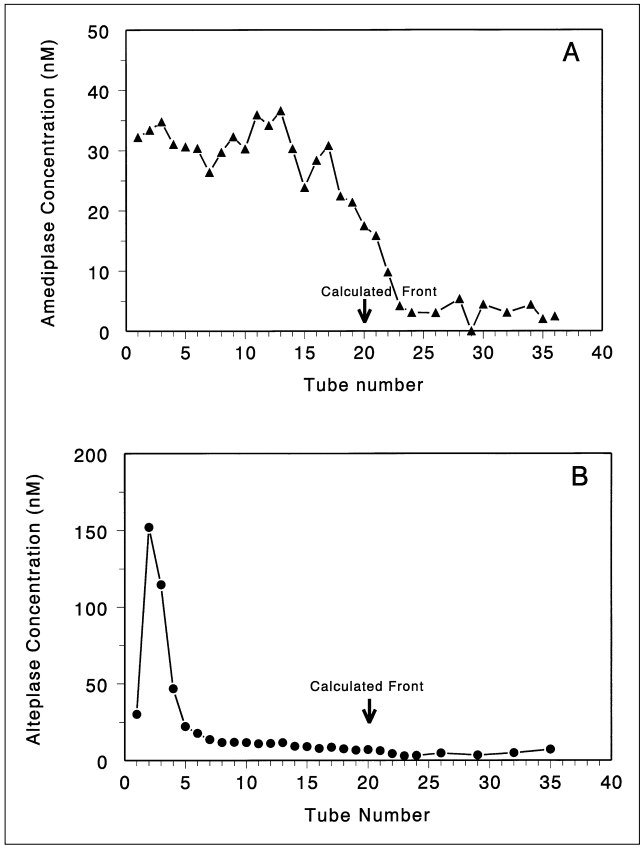

Figure 4: Clot penetration of amediplase (A) or alteplase (B) by fluid permeation. Citrated plasma containing 28 nM (1.2 µg/ml) amediplase or 28 nM (2.0 µg/ml) alteplase was pumped into a syringe containing a recalcified plasma clot under conditions of blocked fibrinolysis. The spatial distributions of the two plasminogen activators (mean of two inde-pendent experiments) are shown.

(3.2 mg/ml final concentration) or citrated plasma, respectively. The mixtures were then clotted with calcium and thrombin in the absence or presence of the fibrinolysis inhibitor aprotinin (Trasylol), incubated at 37°C for 10-15 min and centrifuged. The amounts of unbound plasminogen activator in the supernatants were determined by ELISA assays and compared with the amounts in control mixtures, which were not clotted. The calculated amounts of bound plasminogen activator are presented in Figure 3. In the purified system 40-50% of the amediplase and 94-97% of the alteplase bound to the clots. In control experiments with a lower amediplase concentration (10 ng/ml, i.e. comparable to the molar concentration of alteplase) also 40-50% of the amediplase bound to the clots (not shown). In a plasma system, the extent of clot binding was somewhat lower than in the purified system: 22% for amediplase and 72-77% for alteplase. Amediplase as well as alteplase bound similarly to clots with and without fibrinolysis.

The effect of the lysine-analogue epsilon-aminocaproic acid (EACA, 10 mM, final concentration) on the binding of amediplase and alteplase was studied in the purified system in the presence of Trasylol. EACA completely inhibited fibrin-binding of amediplase and reduced the binding of alteplase from 90 to 70% (not shown).

Clot penetration by fluid permeation

Plasma clots were prepared by recalcification of citrated platelet-poor plasma in syringes and were about 50% permeated with citrated plasma containing either 28 nM (1.2 µg/ml) amediplase or 28 nM (2.0 µg/ml) alteplase, together with aprotinin and heparin to block fibrinolysis and coagulation, respectively. Figures 4A and B show that amediplase did not bind to the

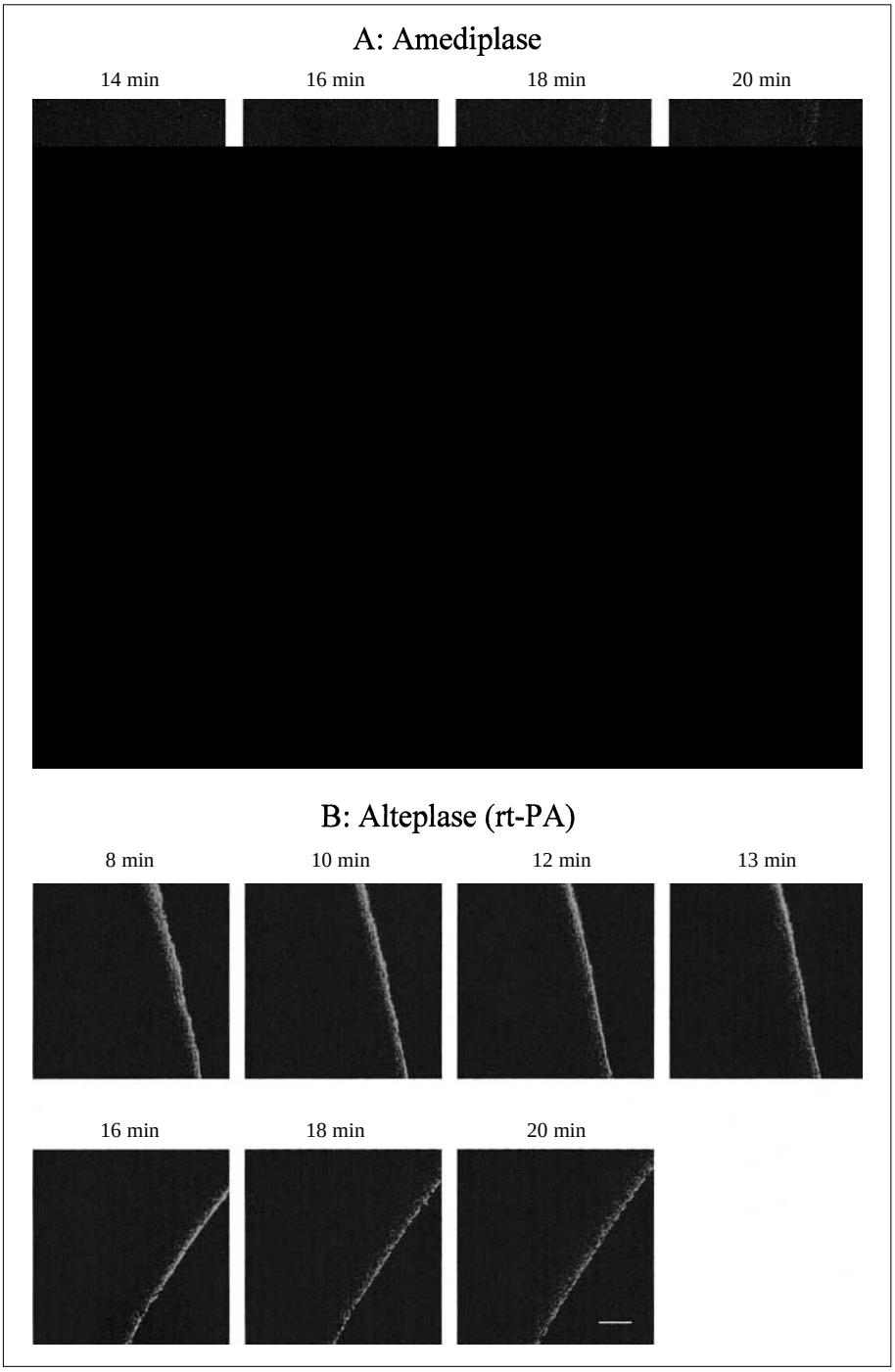

Figure 5: External plasma clot lysis by 1.0 µg/ml amediplase (A), 1.9 µg/ml alteplase (B) or 300 IU/ml two-chain urokinase (C) at room temperature as studied by confocal microscopy. The accumulation of FITC-plasminogen on the fibrin fibres reflects the localisation of the plasminogen activator. Serial images were taken at the indicated time points. The images in B were taken at two separate sites of the clot (from 8-13 min on one site and from 16-20 min on another site). Bar, 40 µm.

fibrin matrix of the clot and penetrated into the clot virtually without hindrance. In contrast, alteplase accumulated in the top part of the clot reaching concentrations of up to 175 nM (6-fold higher than in the permeating fluid). As a result, the alteplase concentrations deeper into the clot were lower than that in the permeating fluid and lower than the amediplase concentrations in corresponding areas in the experiments with amediplase.

Clot penetration by diffusion during plasma clot lysis

The clot penetration of amediplase, alteplase and two-chain urokinase during plasma clot lysis was studied by confocal fluorescence microscopy. Preformed plasma clots containing tracer amounts of FITC-plasminogen were surrounded by plasma containing both FITC-plasminogen and one of the plasminogen activators to induce lysis. The localisation of the

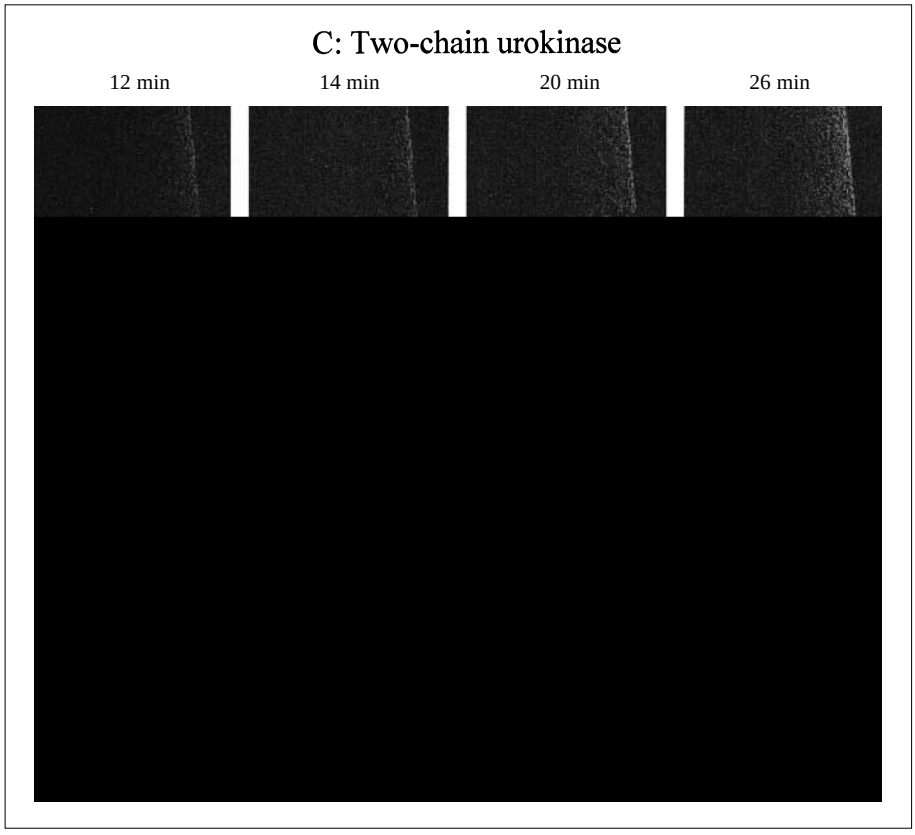

Figure 5: C) Continued

plasminogen activator within the clot is reflected by the accumulation of FITC-plasminogen on the partially nicked fibrin fibres. A high accumulation of FITC-plasminogen on the clot surface does not necessarily reflect accumulation of plasminogen activator (15). Figure 5A shows the results obtained with 1.0 µg/ml amediplase in the plasma. During the first 24 min increasing amounts of amediplase entered the clot, but the clot surface did not yet move. From 26–36 min the clot surface moved as a result of clot lysis. Figure 5B shows the results obtained with 1.9 µg/ml alteplase. Images were taken on two separate sites of the clot surface. Alteplase hardly entered the clot and remained localised on or close to the clot surface. From the beginning of the observation time (at 8 min) the clot surface moved as a result of clot lysis. Figure 5C shows the results obtained with 300 IU/ml two-chain urokinase. The pattern was similar to that of amediplase. During the first 26 min increasing amounts of urokinase entered the clot and the clot surface did not yet move. From 26–46 min the clot surface moved at a more or less constant speed. Figure 6 shows representative images of the three plasminogen activators in order to compare the depth of the penetration of the plasminogen activators. While alteplase was only present near the clot surface, two-chain urokinase entered deeply into the clot. Amediplase behaved more like two-chain urokinase than like alteplase and also entered deeply into the clot. The lag phase in clot lysis by amediplase, also observed in the external lysis shown in Figure 2 particularly at low amediplase concentrations, could be ascribed to the pro-enzyme nature of amediplase as well as to its clot penetration behaviour (i.e. no significant accumulation on the clot surface as occurs with alteplase). The observation that active two-chain urokinase shows a similar lag phase as amediplase in these experiments, indicates that the penetration behaviour plays a dominant role.

Discussion

In this study, we demonstrated that the penetration of amediplase into plasma clots is much stronger than that of alteplase, most likely because amediplase has a lower affinity for fibrin than alteplase.

In the first part of the study, the clot lysis activities of amediplase and alteplase were compared. In internal clot lysis models, in which the plasminogen activators are incorporated into purified fibrin clots or plasma clots, amediplase was about 10 times less active than alteplase. In an external plasma clot lysis model, in which the plasminogen activators are added to the plasma outside the clot, the two plasminogen activators revealed a similar clot lysis activity, in particular at therapeutic concentrations of 1 µg/ml and higher. The lower activity of amediplase is apparently compensated for by the higher clot

penetration. It would be interesting to study if amediplase is even more active than alteplase towards large clots in which the penetration properties are probably more significant.

In the second part of the study the fibrin binding properties of amediplase and alteplase were compared, both in a purified system and in a plasma system. In correspondence with previous studies with K2tu-PA (5), amediplase bound only partially to purified fibrin clots of 3.2 mg/ml (40-50%), while alteplase fully bound to these clots (94-97%). Although clot binding was smaller in a plasma milieu, a similar difference between amediplase and alteplase was observed with plasma clots (22% binding for amediplase and 72-77% binding for alteplase).

The strong fibrin binding of alteplase is ascribed to its finger domain and kringle 2 domain (3, 4). The latter domain harbours a so-called lysine binding site which possibly interacts with an intrachain lysine residue of intact fibrin, probably in the αC-domain (17). Several authors have suggested that alteplase may show an increased fibrin binding during fibrin degradation due to the generation of carboxy-terminal lysine residues (18-20). Because a similar phenomenon might occur with amediplase via its kringle domain, fibrin binding was measured both in the absence and in the presence of Trasylol. However, fibrin binding was similar in clots with and without fibrinolysis. We cannot exclude that increased fibrin binding might happen during fibrin degradation, but the present study indicates that it is not a dominant phenomenon. The results are in line with the observation that the kringle 2 domain of alteplase does not prefer carboxy-terminal lysine residues to intrachain lysine residues for binding (21).

Fibrin binding experiments in the presence of EACA showed that this lysine analogue fully inhibited the binding of amediplase, which indicates that the observed binding was specific and involved the lysine binding site in the kringle of amediplase. This property clearly distinguishes amediplase from urokinase-type plasminogen activator, which does not bind to fibrin.

Many t-PA/u-PA chimeric molecules described in the literature exhibit a lower fibrin binding potency than expected, because of an intramolecular interaction between a positively charged amino acid from u-PA residues 144-157 and the lysine binding site in kringle 2 of the t-PA moiety (22). It is interesting to note that amidiplase does not contain u-PA residues 144-157 and thus exhibits a free lysine binding site.

In the third set of experiments it was demonstrated that permeation-driven penetration of amediplase and alteplase into plasma clots prepared in a syringe differed significantly. While alteplase was concentrated on the top of the plasma clot, amediplase entered the clot without significant hindrance. The moderate binding of amediplase to plasma clots in a static system (22% binding), apparently did not significantly affect the penetration under flow conditions. A similar difference with alteplase was previously observed with reteplase, which is composed of the kringle 2 and the protease domain of alteplase (16).

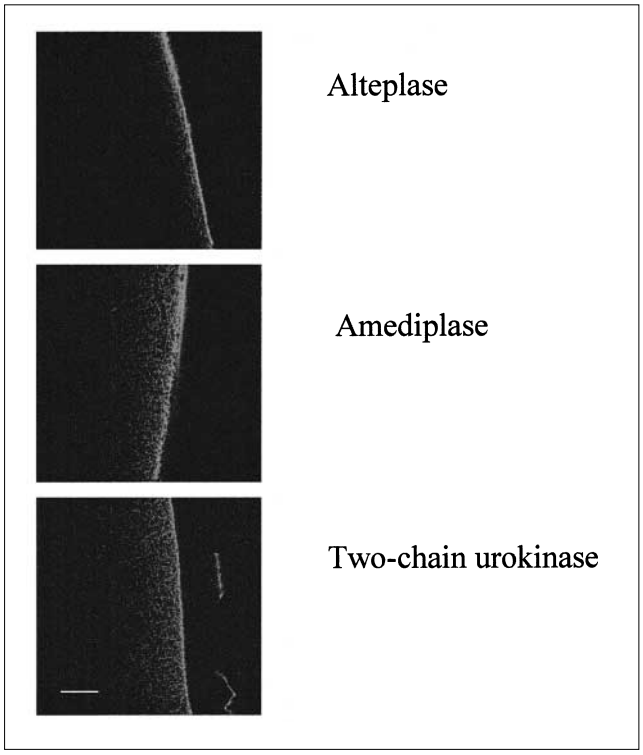

Figure 6: External plasma clot lysis as studied by confocal microscopy. Three representative images from Figures 5A-C (alteplase 12 min, amediplase 30 min and two-chain urokinase 38 min) were combined to compare the depth of penetration of the three plasminogen activators. The images show accumulation of FITC-plasminogen in the inner parts of the clots treated with amediplase or two-chain urokinase, but no accumulation in the inner parts of the clot treated with alteplase. Bar, 40 μm.

In the fourth set of experiments using confocal microscopy we demonstrated that diffusion-driven penetration of amediplase and alteplase into plasma clots during lysis also differed significantly. As observed earlier (15), alteplase was only detected on or close to the clot surface, while two-chain urokinase, which has no affinity for fibrin was also detectable inside the clot. Although the indirect detection system via the accumulation of FITC-labelled plasminogen on fibrin fibres did not allow an accurate assessment of the depth of the penetration, it was clear that amediplase behaved more similarly to two-chain urokinase than to alteplase.

We concluded that the fibrin clot binding of amediplase is smaller than that of alteplase. The clot penetration of amediplase is, probably as a result of a diminished clot binding, stronger than that of alteplase, both under permeation-driven and under diffusion-driven transport conditions. Enhanced clot penetration of amediplase might contribute to its promising efficacy in treating patients with acute myocardial infarction (10).

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