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Transport Phenomena and Clot Dissolving Therapy: An Experimental Investigation of Diffusion-Controlled and Permeation-Enhanced Fibrinolysis

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Summary

We have investigated the effects of diffusive and convective transport on fibrinolysis. Using a constant pressure drop (ΔP/L) from 0 to 3.7 mmHg/cm-clot to drive fluid permeation, various regimes of lytic agents were delivered into fine and coarse fibrin gels (3 mg/ml) and whole blood clots. Using plasmin (1 μM) delivered into pure fibrin or urokinase (1 μM) delivered into glu-plasminogen (2.2 μM)-laden fibrin, the velocity at which a lysis front moved across fibrin was greatly enhanced by increasing ΔP/L. Lysis of fine and coarse fibrin clots by 1 μM plasmin at ΔP/L of 3.67 and 1.835 mmHg/cm-clot, respectively, led to a 12-fold and 16-fold enhancement of the lysis front velocity compared to lysis without pressure-driven permeation. For uPA-mediated lysis of coarse fibrin at ΔP/L = 3.67 mmHg/cm-clot, the velocity of the lysis front was 25-fold faster than the lysis front velocity measured in the absence of permeation. Similar permeation-enhanced phenomenon was seen for the lysis of whole blood clots. Without permeation, the placement of a lytic agent adjacent to a clot boundary led to a reaction front that moved at a velocity dependent on the concentration of plasmin or uPA used. Overall, these studies suggest that transport phenomena within the clot can play a major role in determining the time needed for reperfusion during fibrinolysis.

Introduction

The impact of fluid permeation within the interstitial regions of blood clots during thrombolysis has only recently been characterized (1–5). Although fluid permeation experiments have been used as an experimental tool to study the structure of fibrin gels (6–8), little work has been done to explore the kinetics of fibrinolysis under controlled conditions of transport.

A blood clot is a very heterogeneous entity which has a biochemistry and structure that is highly dependent upon the thrombotic and hemodynamic events which prevail during its formation. With arterial clots which can be retracted by platelets as well as compacted by hemodynamic forces, the occlusion is sufficiently strong to block blood flow. Yet, blood clots may be sufficiently permeable to allow a slow permeation (also termed: advection, convection, perfusion) of fresh fluid through interstitial regions driven by the high pressures of the arterial system. The pressure drop across a coronary occlusion can exceed 60 mmHg/cm-clot (or 8×10^4 dyne/cm^2 per cm-clot). The permeation velocity of this interstitial flow is quite small compared to nominal values of blood flow through open vessels, however this permeating fluid would be expected to deliver thrombolytic agents into the clot much more efficiently than diffusion mechanisms. Brownian motion is very inefficient in transporting proteins across long distances of a few millimeters of a clot. Diffusion coefficients of proteins are very small, typically on the order of 10^{-7} cm^2/s (9). Nonetheless, large molecules such as bovine serum albumin, fibrinogen, or immunoconjugates have been found to enter and move in platelet-retracted clots or plasma clots by diffusion without any steric hindrance due to the presence of the fibrin network (10–11).

Pressure-driven permeation of fluid through a fibrous structure like fibrin is accurately described by Darcy's Law (Eqn. 1) for slow interstitial flow where the volumetric flowrate Q per cross sectional area A is proportional to the pressure drop ΔP/L across the clot (6–8) where k is the specific permeability and μ is the viscosity of the perfusion buffer. Unfortunately, no specific permeability data is available for actual platelet-retracted, flow-compacted blood clots that contain layers of fibrin, platelets, and red blood cells.

Darcy's Law $$v = \frac{Q}{A} = \left(\frac{k}{\mu}\right)\frac{\Delta P}{L}$$ Eqn. 1

For fibrin that is compacted to 5% of its original plasma clot volume (final concentration of 60 mg-fibrin/ml), we estimate the specific permeability to be about 10^{-11} cm^2 or less (4). The permeation velocity through such a structure would be about 0.001 cm/s at pressure drops across the clot of 60 mmHg/cm-clot.

In the absence of permeation, fibrinolysis is severely diffusion limited – much of the plasminogen activator near the clot is wasted due to lack of available plasminogen substrate. Lysis rates measured in vitro with preformed clots in the absence of permeation likely are greatly reduced by diffusional limitations within the clot (even with good mixing of the fluid external to the clot). The kinetic constants derived from these types of experiments are system-dependent values which lump kinetic and diffusional phenomena. In contrast, kinetic studies where enzymes are added to dispersed fibrin (12–13) result in a well mixed system where fibrin solubilization is not limited by the rate of diffusion. Similarly, the fibrin plate assay which uses thin films of fibrin less than a fraction of a millimeter are unlikely to be diffusionally limited since the added enzymes achieve uniform concentrations in the fibrin in a relatively short time. Solubilization rates measured with thin fibrin films yield intrinsic kinetic data without diffusional artifacts, but the kinetic data alone do not allow direct quantitative prediction of reperfusion times under conditions of permeation. Such predictive capability requires estimates of transport processes (2, 4).

We have carried out experimental studies under controlled conditions of perfusion of lytic agents into purified fibrin gels of coarse and

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fine fibers. It was the intent of the present research to demonstrate how the rate of fibrin degradation depends upon the pressure drop across the clot. These experiments demonstrated a dramatic enhancement (often greater than 1,000% increases) of fibrinolysis due to permeation of fibrinolytic mediators into coarse and fine fibrin clots. By evaluating the role of mass transfer during fibrinolysis, relevant information may be acquired for the quantitative prediction and optimization of the time needed for reperfusion of a clot based on intrinsic kinetic and transport data.

Material and Methods

Proteins and Reagents

Purified human thrombin (Sigma) was obtained as a lyophilized powder (specific activity: 3,000 NIH U/mg). The thrombin was dissolved in high purity water at 200 NIH U/ml, aliquoted, and frozen at –75° C. Lyophilized human fibrinogen (Grade L, Kabi AB) was dissolved in 0.05 M tris(hydroxymethyl)-aminomethane (Tris-HCl) pH 7.4 and dialyzed at 4° C against 0.05 M Tris-HCl containing 0.1 or 0.3 M NaCl and 5 mM $CaCl_2$ for 18 h with 1 L changes every 6 h. The solution was centrifuged at 2,000 × g for 20 min (4° C) and the supernatant was frozen in small aliquots at –75° C. Purified human glu-plasminogen and human plasmin (American Diagnostica Inc.) were reconstituted, centrifuged at 2,000 × g for 20 min (4° C) and stored at –75° C. Recombinant urokinase was obtained as a gift from Dr. A. Sasahara (Abbott Laboratories). The urokinase was dissolved to give a concentration of 50,000 IU per ml of urokinase activity. A μM solution of urokinase was prepared by dilution of this stock immediately prior to lysis experiments. Human venous blood was collected without anticoagulation by venipuncture from healthy, nonsmoking volunteers.

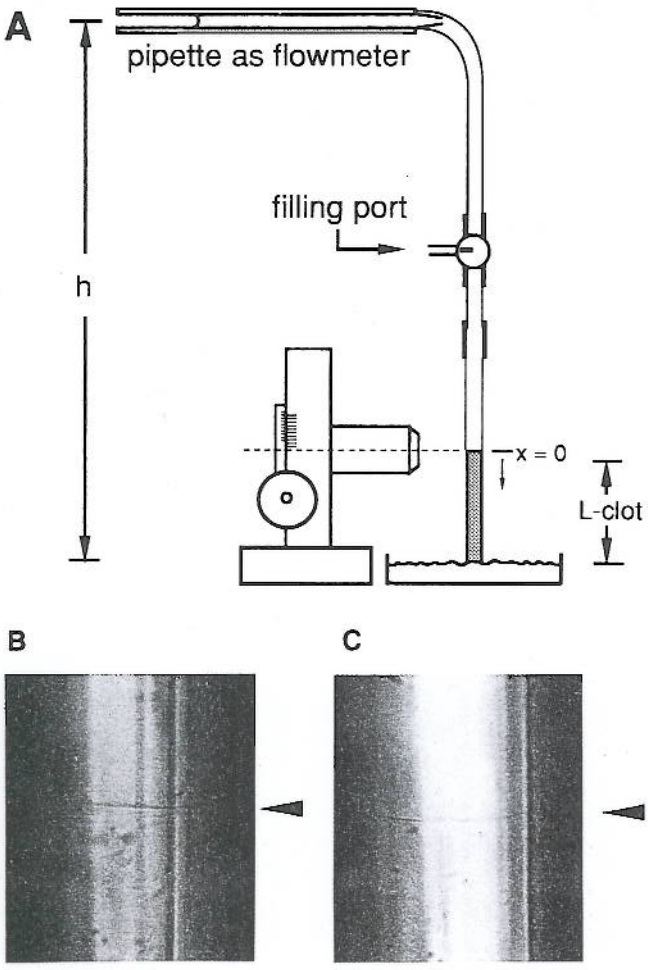

Fig. 1 Experimental configurations to study the lytic fronts moving through preformed clots (A). Fibrin clots formed in 100 μl capillaries were 2.0 ± 0.1 cm long. Whole blood clots formed in 1 ml polystyrene pipettes were 4.0 ± 0.1 cm long. The capillaries were backfilled with lytic solution and a pressure head of 1.0 to 20.0 cm-H_2O was immediately attached to the capillary at the start of the experiment. Alternatively for diffusion experiments, capillaries were sealed at each end and held horizontally to eliminate pressure gradients. Up to six gels could be run simultaneously. The macroscopically observable fluid/fibrin interface in capillaries with a fine fibrin gel (B) and a coarse fibrin gel (C) was located and tracked by visual observation

Preparation of Fibrin Gels and Whole Blood Clots

All operations were carried out at 37° C in an environmental room with freshly thawed proteins which were incubated at 37° C for 20 min prior to use. Clots of purified human fibrinogen (final concentration, 3 mg/ml) were formed in glass capillary tubes (1.5 mm ID) after rapidly and uniformly mixing 1:1 dilutions of a buffered fibrinogen solution with buffered thrombin (final concentration, 1 U/ml). The buffer for fibrin polymerization was 0.05 M Tris-HCl, 5 mM $CaCl_2$ (pH 7.4) with 0.1 M NaCl or 0.3 M NaCl to obtain turbid coarse gels and transparent fine gels, respectively. Prior to lysis experiments, the fibrin gels were allowed to set for over 10 h to achieve complete polymerization. Fibrin gels were 2 cm long. In some experiments, glu-plasminogen was preincubated with the fibrinogen at 37° C for 20 min before addition of thrombin (final concentration, 2.2 μM glu-plg). Fibrin gels in tubes showing visible heterogeneity or other flaws were discarded. For lysis experiments with whole blood clots, freshly drawn, nonanticoagulated blood was immediately backfilled into sterile 1 ml polystyrene pipettes (3 mm ID) to yield 4 cm long clots. The blood samples were allowed to clot for 120 min at 37° C.

Clot Lysis Experiments

The experimental configuration for permeation studies of clot lysis was modified from Carr (6–7) as shown in Fig. 1. Purified fibrin clots or whole blood clots were lysed with a 50 μl addition of lytic enzyme that was carefully loaded above the clot. The tube containing the fibrin clot was connected using Tygon tubing to a horizontally mounted 1 ml pipette filled with 0.05 M Tris-HCl buffer to achieve a hydrostatic pressure head across the clot from 1 to 20 cm-H_2O. Degradation of the clot was monitored within 5 min of addition of plasmin or urokinase. Volumetric flowrate was read from the calibrated horizontal pipette. Clot lysis was expressed as the reduction in gel length over time (mm/min). In diffusion-controlled lysis experiments (i.e. at zero pressure drop), clots were lysed by loading 50 μl of enzyme solution adjacent to the clot. The capillary was then sealed with vacuum grease at both ends to eliminate any macroscopic convective flow and held horizontally during the experiment. Changes in the position of fluid-gel interface were recorded with time as the clot lysed. Introduction of a Coomassie blue solution adjacent to the fibrin created an exceedingly sharp interface with no visible penetration of the dye into clot at early times. Thus, the introduction of the fibrinolytic media adjacent to the polymerized fibrin gel at the start of the experiment did not cause any significant introduction of the enzymes into the fibrin by convection.

Microscopy

A double coverslip chamber using two coverslips separated by a parafilm gasket provided a parallel plate geometry for the creation of fibrin gels. A Leitz Aristoplan differential interference contrast (DIC) microscope with 1.32 NA planapo 100 X oil immersion objective lens and 1.4 NA oil immersion condenser was used to visualize the fibers of coarse fibrin gels. Dynamic experiments were visualized with a CCD72 (Dage-MTI) video camera with realtime digital contrast enhancement and background subtraction using an Argus 10 image processor (Hamumatsu). Subsequent analysis of video images was conducted on a Macintosh IIci with a Quickcapture frame grabber (Data Translation) and NIH Image 1.49 software.

Results

Two types of experiments were performed. The objective of the first set of experiments was to investigate the accessibility of the interstitial regions of the fibrin under conditions of pressure-driven permeation. The objective of the second set of experiments was to investigate the concentration dependence of fibrinolysis under conditions of diffusional transport only. In our experiments, fibrinolysis was measured by the reduction of fibrin gel length with respect to time. The velocity of the lysis front, v is precisely related to the solubilization rate (S, fibrin solubilized per unit time) by $S = A\, C_{fibrin}\, (v)$ where A is the cross sectional area of the clot which has an initial concentration of fibrin, C_{fibrin}.

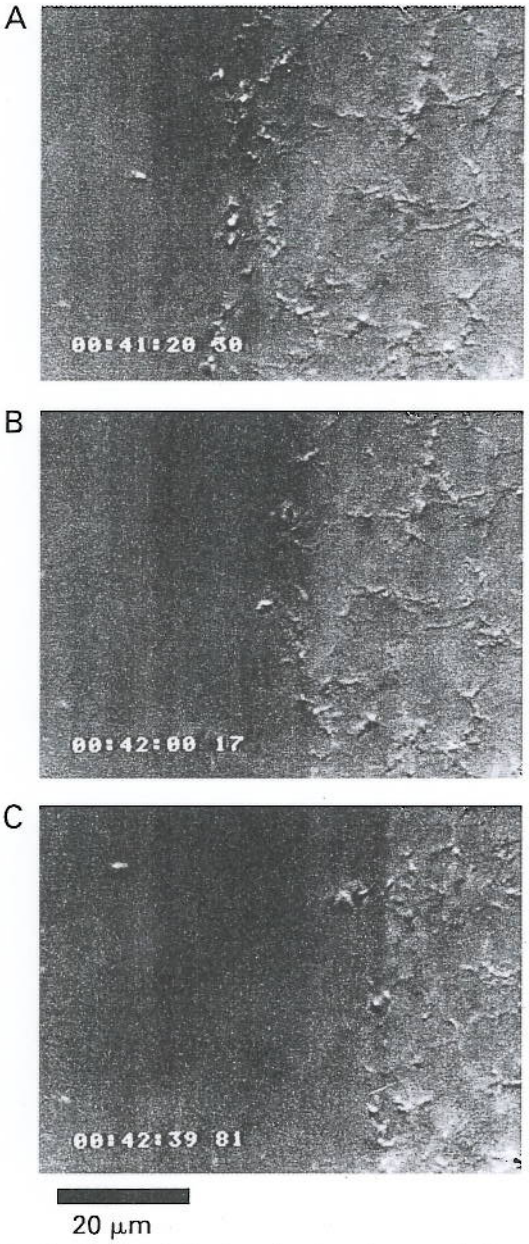

Fig. 2 Microscopically sharp interfaces between fluid and fibrin were visualized with contrast-enhanced 100X DIC microscopy for coarse fibrin gels undergoing lysis. Plasmin (1 μM) was placed adjacent to preformed fibrin (3 mg/ml) and lytic fronts that moved at constant velocity were observed for over 20 min. After several minutes of stable lysis, the lytic front which moved at a velocity of 0.0167 ± 0.0011 mm/min is shown at a reference location (A), 40 s later (B) and 80 s later (C). The zone of dissolving fibrin is less than 5 μm thick in these experiments

It was possible to track the precise position of the lysis front by visual observation. Sharp lysis fronts persisted during the entire experimental regime, and are shown in Figs. 1 and 2. During microscopic observation of coarse fibrin gels undergoing lysis, lysis zones were observed that were exceedingly sharp. As plasmin (1 μM) in free solution came in contact with the fibrin boundary, the fibers at the boundary displayed Brownian motion as their linkages to other fibers were dissolved (Fig. 2). A few microns deeper into the fibrin, the fibers were completely rigid and lacked any thermal motion. Plasmin, although freely diffusible in the large liquid filled interstitial regions of the clot (10, 11, 14, 15), was evidently bound rapidly to the solid nonmobile fibers of fibrin (16) leading to a sharp front.

Plasmin-mediated Lysis of Fibrin Gels under Conditions of Inner Clot Permeation

We perfused Tris-HCl buffer containing 1 μM plasmin through purified fine and coarse fibrin gels (created either in 0.3 M NaCl or in 0.1 M NaCl Tris-HCl solution) using pressures drops from 0 to 3.67 mmHg/cm-gel (10 cm-H_2O/2.0 cm-gel). After an initial transients, the lysis front proceeded at a constant velocity, allowing a determination of the velocity by a linear fit of the interface displacement with time. A steeper slope indicated a faster lysis front velocity and a faster fibrin solubilization rate. Fine gels (2 cm in length) completely lysed within 100 min at a pressure drop of 3.67 mmHg/cm whereas over 20 h was required for complete lysis of the fine gel in the absence of permeation. Thus, a 12-fold increase in lysis rate was observed when fibrinolysis occurred under conditions of pressure driven permeation (Fig. 3). The liquid/fibrin interface did not move when gels were placed under a pressure drop without plasmin in the permeation fluid. In experiments with plasmin-mediated lysis of coarse gels, we observed similar large enhancements due to permeation (Fig. 4). A nearly 16-fold enhancement in lysis rate occurred with a pressure drop of 1.835 mmHg/cm-clot when compared to the lysis measured in the absence of permeation.

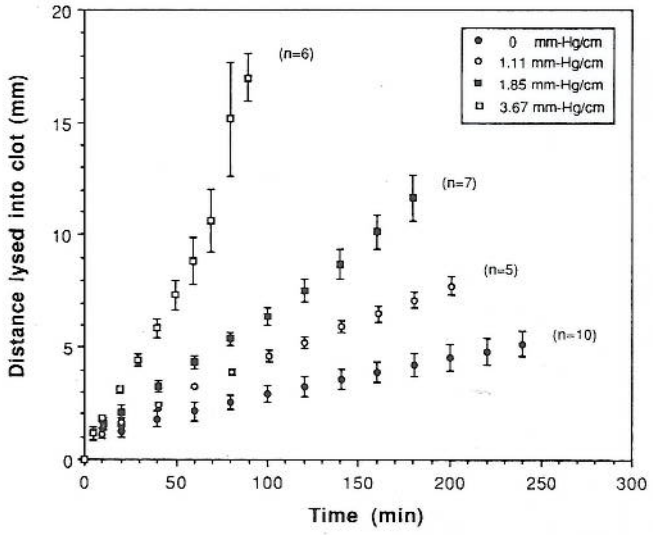

Fig. 3 The effect of pressure drop on plasmin-mediated lysis of fine fibrin gels. Fibrinogen (3.0 mg/ml) was polymerized with 1 U/ml thrombin in 0.3 M NaCl. Plasmin (1 μM) was then placed on top of the gel and introduced into the fibrin at constant pressure drops of 0 to 3.67 mmHg/cm-clot (10 cm-H_2O/2 cm-clot). The position of the lytic front was monitored with time as well as the perfusion media flow rate (n, replicate gels)

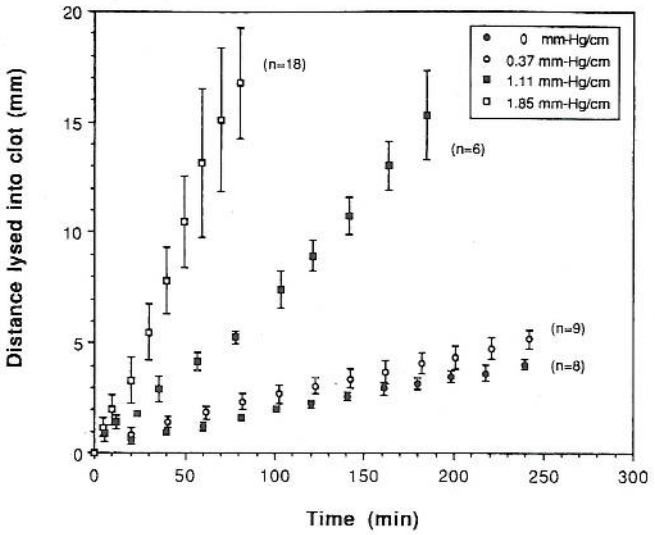

Fig. 4 The effect of pressure drop on plasmin-mediated lysis of coarse fibrin gels. Fibrinogen (3.0 mg/ml) was polymerized with 1 U/ml thrombin in 0.1 M NaCl. Plasmin (1 μM) was then placed on top of the coarse gel and introduced into the fibrin at constant pressure drops of 0 to 1.835 mmHg/cm-clot (5 cm-H_2O/2 cm-clot). The position of the lytic front was monitored with time as well as the perfusion media flow rate

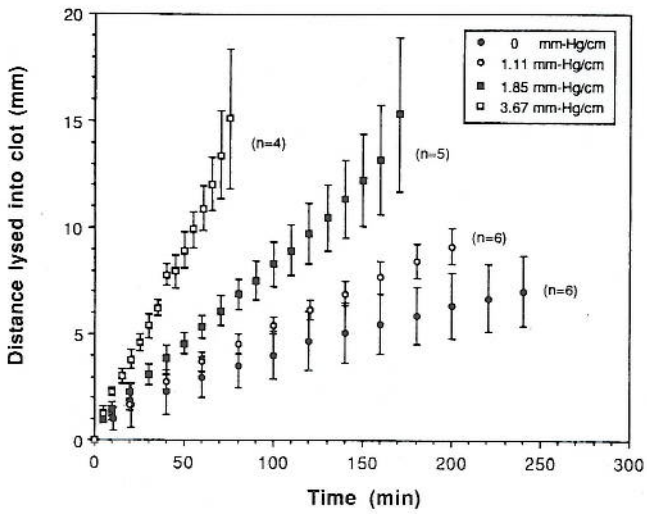

Fig. 6 The effect of pressure drop on the uPA-mediated lysis of fine fibrin gels. Fibrinogen (3.0 mg/ml) and 2.2 μM glu-plasminogen were polymerized with 1 U/ml thrombin in 0.3 M NaCl. Urokinase (1 μM) was then placed on top of the gel and introduced into the fibrin at constant pressure drops of 0 to 3.67 mmHg/cm-clot (10 cm-H_2O/2 cm-clot). The position of the lytic front was monitored with time as well as the perfusion media flow rate

For a given pressure drop, coarse gels required less time for complete lysis compared to the time required to lyse fine gels because the coarse gels were more permeable (higher specific permeability) to flow. For example, the lysis front velocity of coarse gels was about four times faster than that of fine gels at a pressure drop of 1.835 mmHg/cm, while the lysis rate of coarse gels was only two times faster than the lysis rate of fine gels at a $\Delta P/L$ = 1.11 mmHg/cm. To compensate for the fact that coarse gels were more permeable to flow than fine gels, the permeation velocity for each lysis experiment was calculated from the perfusion flow rate (Eqn. 1). Fig. 5 shows the relation between the velocity of the lysis front and the velocity of the perfusion fluid through fine and coarse fibrin. A highly linear correlation was obtained for plasmin-mediated lysis of either fine or coarse gels. The velocity of the lysis front was 0.75 times the fluid permeation velocity (range: 0 to 0.6 mm/min) for plasmin-mediated lysis of either fine or coarse fibrin. This relationship demonstrated that enhanced delivery of plasmin into the clots by permeation reduced diffusional limitations. In these experiments, we found that fine and coarse gels lysed at essentially the same rate when plasmin (1 μM) permeated into the gels at a given velocity.

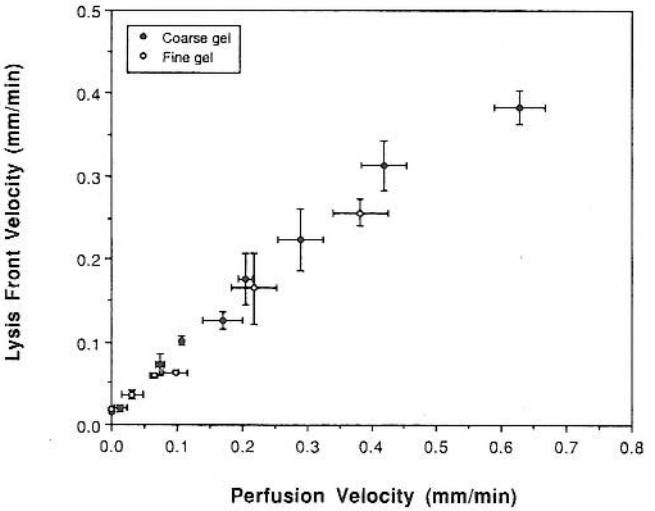

Fig. 5 The velocity of the plasmin-mediated lysis front was directly related to the velocity of the perfusion fluid through the clot. The velocity of the lytic front (dx/dt) was calculated from Figs. 3 and 4 at various times during each experiment. The perfusion velocity was derived from the volumetric flowrate which had been measured throughout the experiment

Urokinase-mediated Lysis of Fibrin Gels under Conditions of Permeation

Experiments for urokinase-mediated lysis were repeated under similar conditions as previously described, except that glu-plasminogen was incorporated into the fibrin during fibrinogen polymerization with thrombin. Perfusion buffer containing 1 μM urokinase was used for the permeation experiments. Figs. 6 and 7 show the effect of urokinase permeation for fine and coarse clots, respectively. The time needed for reperfusion of a 2-cm long coarse fibrin gel was markedly reduced to less than 10 min at a pressure drop of 3.67 mmHg/cm-clot. This was a 25-fold enhancement compared to the lysis velocity of coarse gels in the absence of permeation. Similar to our findings with plasmin-mediated lysis, coarse gels lysed several fold faster (6.5 fold at $\Delta P/L$ = 3.67 mmHg/cm) than fine gels exposed to 1 μM uPA at the same pressure drop. This was due to the higher specific permeability of coarse gels. To correct for the fact that coarse gels were more permeable than fine gels, we calculated the permeation velocity for each lysis experiment. We found that the velocity of the lysis front strongly correlated with the velocity of the permeating fluid (Fig. 8). There was no marked difference between the lysis rates of fine and coarse clots when exposed to 1.0 μM uPA under conditions of permeation.

Plasmin and uPA-mediated Lysis of Purified Fibrin Clots in the Absence of Permeation

In the absence of fluid permeation, the effect of various concentrations of plasmin solution (0.01 to 10 μM) placed adjacent to the fibrin was examined. As shown in Figs. 9A and 9B for lysis of fine and

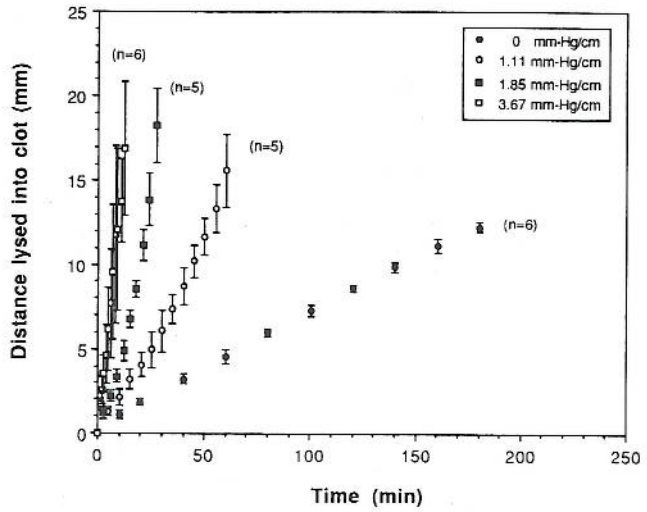

Fig. 7 The effect of pressure drop on the uPA-mediated lysis of coarse fibrin gels. Fibrinogen (3.0 mg/ml) and 2.2 μM glu-plasminogen were polymerized with 1 U/ml thrombin in 0.1 M NaCl. Urokinase (1 μM) was then placed on top of the coarse gel and introduced into the fibrin at constant pressure drops of 0 to 1.835 mmHg/cm-clot (5 cm-H_2O/2 cm-clot). The position of the lytic front was monitored with time as well as the perfusion media flow rate

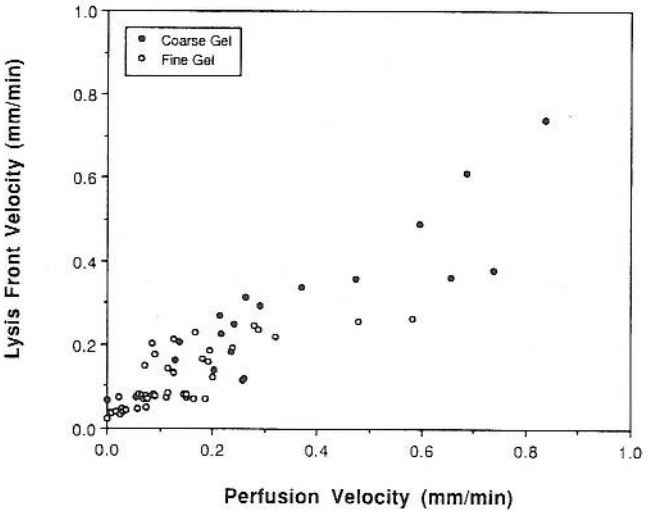

Fig. 8 The velocity of the urokinase-mediated lysis front was directly related to the velocity of the perfusion fluid through the clot. The velocity of the lysis front (dx/dt) was calculated from Figs. 6 and 7 at various times during each experiment. The perfusion velocity was derived from the volumetric flowrate which had been measured throughout the experiment

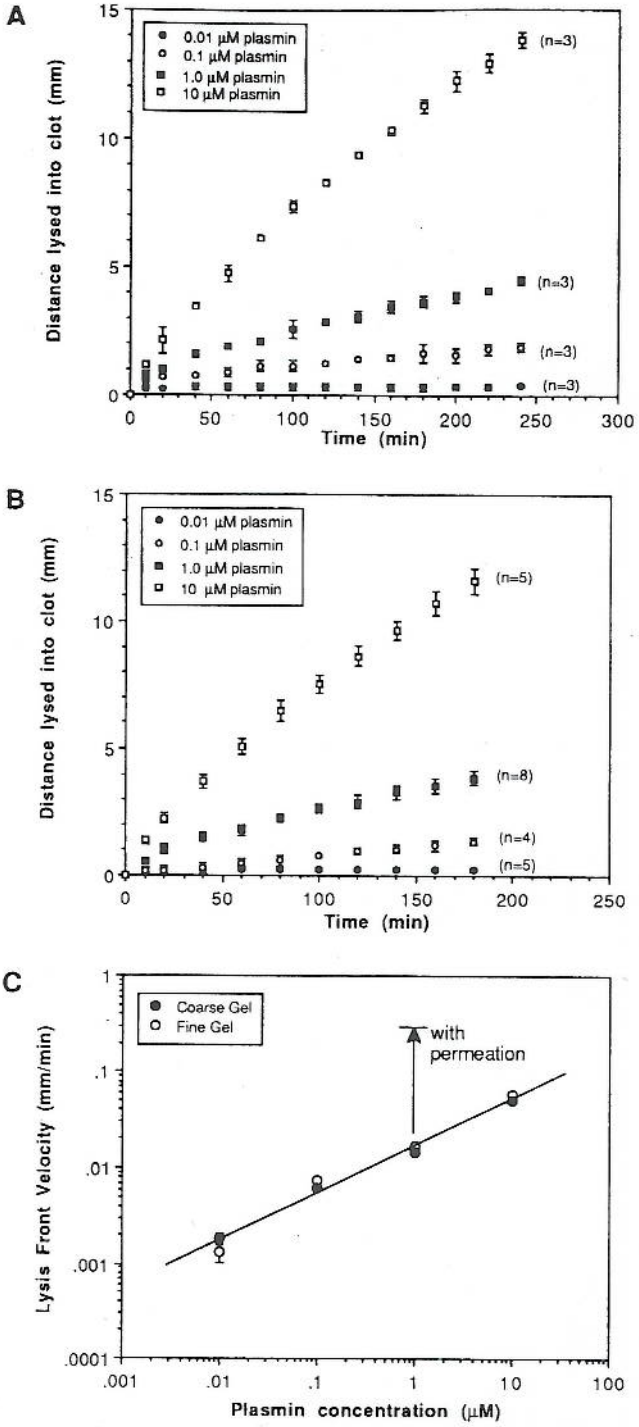

Fig. 9 Under conditions of diffusion-mediated transport (no permeation, $\Delta P/L = 0$), the fluid/fibrin interface moved at a rate which was enhanced by the concentration of plasmin in the solution placed adjacent to the fine (A) or coarse fibrin (B). The lysis front velocity increased as the concentration of plasmin was increased (C). For comparison, lysis velocities are shown for plasmin (1 μM) driven by a pressure drop as indicated

coarse fibrin, a steady state lysis front velocity was achieved after a short transients. Lysis rates were determined by linear fit of experimental data taken after the initial transient period. Higher lysis front velocities for fine and coarse gels were observed as the concentration of plasmin was increased. We found no significant difference in the lysis of fine and coarse fibrin when plasmin was added exogenously to preformed gels (Fig. 9C) in the absence of permeation. For comparison, the range of lysis front velocity for the previously described permeation experiments with 1 μM plasmin are shown in Fig. 9C demonstrating the large enhancement of lysis due to permeation.

In studies similar to those with plasmin, the effect of urokinase concentration on glu-plasminogen-laden fibrin clots was assessed. Urokinase at concentrations ranging from 0.001 to 80 μM was loaded adjacent to glu-plasminogen-laden fibrin. Figs. 10A and 10B show the digestion of fine and coarse fibrin gels, respectively, with time in the absence of permeation. Lysis of fibrin clots was strongly dependent on the urokinase concentration. Fine gels lysed significantly slower than coarse gels at lower urokinase concentrations, but fine and coarse gels lysed equally fast at uPA concentrations higher than 1 μM (Fig. 10C).

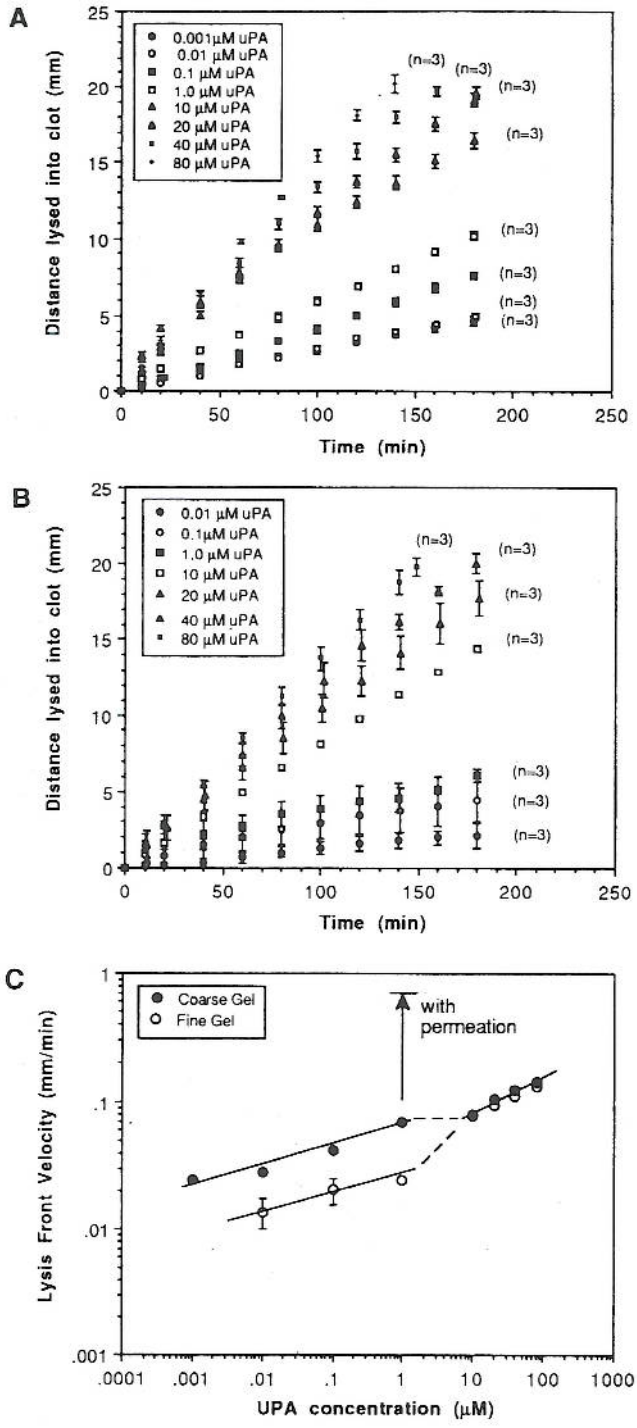

Fig. 10 Under conditions of diffusion-mediated transport (no permeation, $\Delta P/L = 0$), the fluid/fibrin interface moved at a rate which was enhanced by the concentration of urokinase in the solution placed adjacent to the coarse (A) or fine fibrin (B). Glu-plasminogen was present during the polymerization of fibrin. The lysis front velocity increased as the concentration of urokinase was increased (C). In comparison, lysis velocities are shown for uPA (1 μM) driven by a pressure drop as indicated

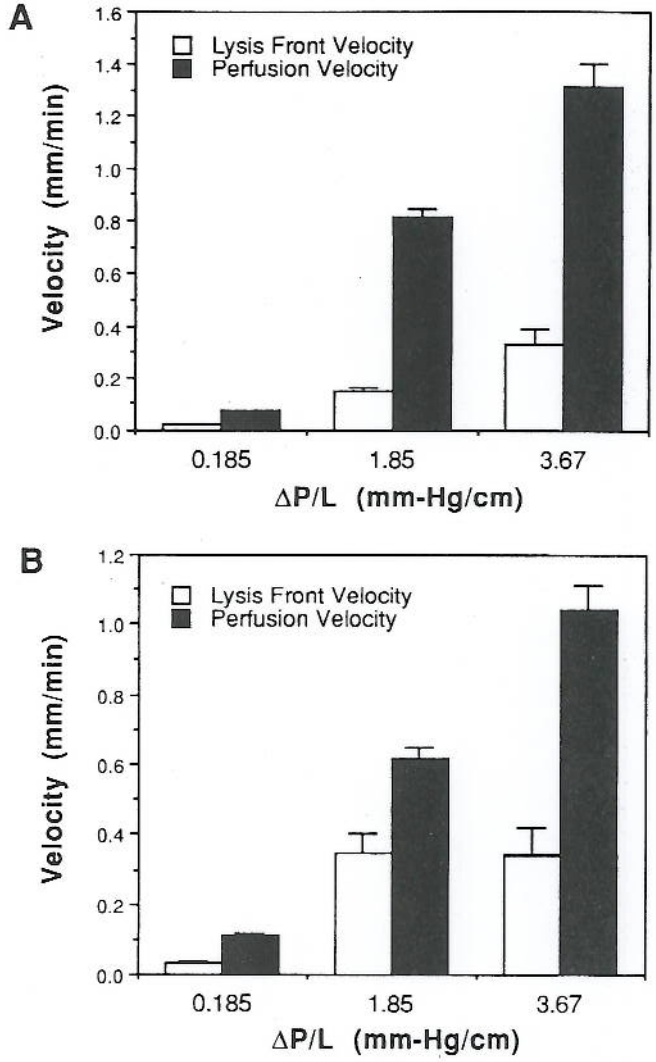

Fig. 11 The effect of pressure drop on plasmin-mediated (A) or uPA-mediated (B) thrombolysis of whole blood clots. Non anticoagulated blood was allowed to clot in polystyrene tubes for 2 h prior to administration of 1 μM plasmin or 10 μM uPA at pressure drops ranging from 0.185 to 3.67 mmHg/cm-clot

Urokinase and Plasmin-mediated Lysis of Whole Blood Clot in the Presence of Permeation

Given the large impact of permeation on lysis of purified fibrin gels, we investigated if these transport mechanisms were also important during thrombolysis of whole blood clots. Whole blood clots were allowed to stabilize for 2 h before the administration of a thrombolytic regime. The choice of 1 ml polystyrene pipettes (3 mm ID) provided for strong clot adhesion to the polystyrene with minimal change in clot volume due to platelet retraction. As expected from Eqn. 1, we found that the rate of permeation of fluid increased as the pressure drop across the clot increased. Permeation of fluid through interstitial regions of the clot caused a slight elution of red blood cells from the bottom of the clot, similar to the findings of Carr (7), however in the absence of plasminogen activators, permeation did not cause the clot to collapse or change volume. The delivery of 1 μM of plasmin into 2 h old clots at a pressure drop of 3.67 mmHg/cm-clot caused a marked 10-fold enhancement of the lysis front velocity as compared to lysis at $\Delta P/L$ = 0.1848 mmHg/cm-clot (Fig. 11A). Similar large enhancements of the lysis front velocity were seen when uPA (10 μM) was delivered into the clot by a pressure drop (Fig. 11B). Thus, pressure driven permeation of thrombolytic agents into preformed whole blood clots provided for a large enhancement of lysis. Permeation allows the full activity of the thrombolytic enzymes to be utilized by significantly reducing the diffusional limitations which starve the enzymes of substrate.

Discussion

In these studies, we have attempted to quantify the effects of mass transfer on fibrinolysis. In one set of experiments, fibrin clots were dissolved by perfusion of enzymes into the clots. In a separate set of experiments, fibrin clots were dissolved with various enzyme concentrations by diffusive delivery of the enzymes into preformed clots. Our studies have shown that permeation of plasmin or urokinase into preformed clots led to a dramatic enhancement of fibrinolysis. Our results with urokinase delivery into purified fibrin gels and unretracted whole blood clots confirm and extend the original observations by Blinc et al. (3, 5). Without permeation, the dissolution of fibrin is controlled by the rate at which plasmin or plasminogen activator can reach suitable substrates for catalysis. Under these conditions, the reaction is diffusion-limited.

The fibrin gels and whole blood clots used in these experiments were created so as to have uniform properties throughout the entire clot structure. Clots were discarded if they had observable density variations due to inadequate mixing and subsequent heterogeneity in polymerization. Such clots had unusually high permeabilities. In real blood clots formed under flow conditions, various fibrin rich and platelet rich layers can occur (lines of Zahn). Large variations in inner clot permeation velocities would be expected as the permeating fluid seeks the path of least resistance. In fact, such heterogeneity in flow has been described by Blinc et al. (3, 5) using NMR visualization of permeation fronts and solute movement in clots. The intent of the present study with uniform clots was to explore the coupling of transport processes and the kinetics of fibrinolysis. In our experience with clotting of whole blood under stationary conditions in vitro, the probability of uniform fibrin polymerization increases as the cross sectional area of the clot decreases. This is due to the attenuation of platelet retraction of domains within the clot because of increased surface to volume ratio found with small diameter tubes. This is consistent with the observations by Blinc et al. (3). Whole blood clots formed in large diameter tubes or formed under flow conditions may have significant variations in fibrin density and permeability that lead to preferred channels of inner clot liquid flow under conditions of pressure driven permeation.

The channeling that leads to clot cannulation described by Blinc et al. (3) may be due to several mechanisms in addition to porosity heterogeneity resulting from variations in platelet retraction. In describing permeation through permeable materials, the Brinkman correction to Darcy's law accounts for the no-slip boundary condition at the wall. Thus, permeation velocities are quite small near the wall (zero velocity at the wall) as compared to the velocities in the middle of the clot.

In our experiments without permeation, the lysis of fibrin and the diffusion of enzymes into the fibrin was essentially a one dimensional transport process in the direction of lysis. At the fluid/fibrin interface, a steep concentration gradient drove a diffusive flux of species into the fibrin. Such transport is well understood theoretically (17, 18), but becomes much more difficult to describe when the interface moves as species diffuse (or convect) through the interface (17, 19). From experiments with fluorescently-labeled albumin, we have not observed the interface to offer resistance to albumin transport in the absence of lysis. This was consistent with the findings of Matveyev et al. and Park et al. (10–11) that large non fibrin-binding proteins can travel freely through a fibrin matrix without any steric hindrance. However, species that bind fibrin such as tPA, plasminogen, or plasmin may rapidly adsorb to the fibers and thus become immobile.

In our high resolution imaging of plasmin or uPA-mediated lysis fronts moving across coarse gels (for example see Fig. 2), some agglomerated material was visible at the interface. It is possible that this layer of material may offer resistance to transport. From microscopic observation of dissolving fibers in fibrin, we did not observe any residual stress in fibrin gels causing retraction as the gels lysed. This was consistent with the fact that the gels were polymerized under stationary, isotropic conditions.

In our experiments without permeation, the velocity of the lytic front was found to be dependent on the concentration of enzyme placed adjacent to the fibrin (Figs. 9 and 10). We believe this is due to two effects: (i) higher concentrations of enzyme provide for a larger concentration gradient to drive diffusive fluxes, and (ii) during moving boundary reaction phenomena with phase change (17, 19), the rate of dissolution of fibrin was proportional to the amount of enzyme at the interface. We suggest that in moving boundary systems with phase change (solid clot to liquid) where species can adsorb to the fibrin, the dissolving interface provided a mechanism by which large concentration gradients were maintained for prolonged periods of time. These steep concentration gradients may provide for diffusive fluxes in great excess of the rates of transport predicted by diffusion under equilibrium conditions without concentration gradients. Brownian motion of proteins in one dimension under gradientless conditions is described by the Einstein equation ($\langle x^2 \rangle = 2\,D$t). Hundreds of minutes would be required for proteins to move just a few millimeters. Yet, the lysis front moved this distance in under an hour. The analytical solution (17) of the one dimensional transient diffusion equation $\{\partial C_i/\partial t = D_i \cdot \partial^2 C_i/\partial x^2\}$ for the fibrin domain where the concentration of the i^{th} species C_i is maintained constant at the inlet boundary ($C_i|_{x=0} = 10\ \mu M$) also indicates that diffusion is a very slow mechanism to move protein through fibrin if the fibrin boundary does not move. In this mathematical formulation where C_i is initially zero in the fibrin and the clot inlet is fixed in space, about 140 min was predicted for a protein like plasmin to reach a concentration of 0.01 μM at a distance of 3 mm into the fibrin. To accurately predict the lysis front velocity (that exceeded 0.1 mm/min in some experiments) will require a moving boundary analysis (17, 19) along with detailed knowledge of the rates of plasmin adsorption/desorption to fibrin as well as the plasmin-dependent rate of fibrin solubilization and the plasmin release rate from dissolving fibers.

In microscopic observations of the lysis front, fibers underwent a complicated transformation due to the action of plasmin (Fig. 2). Current understanding of the structural changes of fibrin during lysis are in complete (4, 20, 21). One view is that fibers of fibrin can undergo radially inward directed lysis i.e. the fiber diameter is reduced as lysis proceeds. Alternatively, fibers may undergo substantial reduction of inner fiber content due to lysis within the fiber (20). Also some swelling of the fiber may be possible. These possibilities are not necessarily exclusive. Using contrast-enhanced microscopy, we have observed a complex phenomena which may be a combination of all these events. We found that fibers in coarse non-crosslinked fibrin underwent an agglomeration process during lysis by which mobile fibers "melted" into one another to form thick "globules" of protein. These globules moved with the front and dissolved as new globules of fibrin appeared. This may be due to the affinity of fibrin fibers for each other which, once the fibers were free to move, caused the fibers to agglomerate. When fibers are constrained via adsorption to a glass coverslip, the fibers do not agglomerate during lysis, but rather disappear by reduction of their radius (Wu and Diamond, data not shown).

In several studies, fine fibrin clots have been reported to be less susceptible to lysis than coarse clots (20, 21). This result is not necessarily expected since more fibrin protofibrils of a fine clot are sterically available to fibrinolytic enzymes as compared to coarse clots in which most of the protofibrils are buried in the interior of the fibrin fibers, possibly

with reduced steric accessibility. Gabriel et al. (21) proposed that enzymes like tPA, plasminogen, and plasmin have difficulty binding and forming strong complexes with single protofibrils. Many of these previous studies have incorporated fibrinolytic mediators (tPA, plasminogen, or plasmin) directly into the polymerizing fibrinogen solution. The experiments described in the present work relied upon introduction of fibrinolytic mediators into performed clots, thus decoupling gelation from fibrinolysis.

We have observed that fine gels were slower to lyse only when low concentrations of uPA were allowed to diffuse into gels polymerized with glu-plasminogen (Fig. 10). At higher concentrations of uPA, any differences in lytic susceptibility of fine and coarse gels were exceedingly small. Similar experiments conducted by diffusion of plasmin into preformed coarse and fine gels showed no significant differences in lytic susceptibility (Fig. 9C). Since uPA activates plasminogen in a fibrin-independent fashion and no differences were observed in the action of plasmin on fine or coarse gels (Fig. 9C), it is possible that thick fibers may serve as a reservoir to create microdomains of high concentrations of plasminogen entrapped during polymerization. This high concentration of plasminogen would then drive the plasminogen activation reaction (22) at faster rates. It is also possible that uPA diffusion is slightly hindered in highly reticulated fine gels (7), however this hindrance would also reduce plasmin mobility which was not seen in Fig. 9C. In the absence of permeation, differences of lytic susceptibility of fine and coarse fibrin were observed for uPA-mediated lysis but were not apparent for plasmin-mediated lysis. Under conditions of robust fibrinolysis at high plasmin or uPA concentrations, no significant differences were found. Also, under conditions of permeation with relatively high concentrations of lytic agents, no large differences were seen between the lysis rates of fine and coarse gels perfused at the same permeation velocity (Figs. 5 and 8). At this point, a full understanding of the distinctions between lysis of fine and coarse fibrin is not possible, but we suggest that the mode by which enzymes are introduced into the clot will affect the mechanisms and kinetics of the process.

Overall, transport phenomena had a dramatic impact on the rate at which clots dissolved. In the arterial system, a large pressure drop across occlusive thrombi can drive permeation if the clot is sufficiently permeable. Even the slowest of inner clot permeation rates would lead to a significant enhancement of the time to reperfuse a clot. If a clot is so dense as to allow essentially no permeation or when in the venous system the pressure drop across the clot is low, the time to reperfuse the clot is controlled by diffusive processes. Rate limiting steps such as the sluggishness of diffusion to transport proteins long distances will be difficult to overcome by protein engineering of new thrombolytic agents. We suggest that in the absence of inner clot permeation, diffusional transport of enzymes into the clot is the rate-limiting step which controls the time to reperfuse a clot during thrombolytic therapy.

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