Plasminogen Binding to Rat Hepatocytes in Primary Culture and to Thin Slices of Rat Liver

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Human $^{125}$I-plasminogen bound readily to rat hepatocytes in primary culture at 4°C and at 37°C. Binding was inhibited by lysine and reversed by lysine, e-aminocaproic acid, or nonradioabeled plasminogen. The $K_d$ for binding of $^{125}$I-plasminogen to hepatocytes was $0.59 \pm 0.16 \text{ mol/L}$, as determined from the saturation isotherm by nonlinear regression ($r^2 = 0.99$) and the Scatchard transformation by linear regression ($r^2 = 0.93$). The number of sites per cell was $14.1 \pm 1.1 \times 10^4$. Fibrinogen synthesis and secretion by hepatocytes was insufficient to account for the major fraction of plasminogen binding, as determined by enzyme-linked immunosorbent assay (ELISA). Polycrylamide gel electrophoresis and trichloroacetic acid precipitation studies demonstrated that plasminogen is neither activated nor degraded when bound to hepatocytes at 37°C. Thin slices of whole rat liver (500 $\mu$m), isolated and prepared totally at 4°C, bound $^{125}$I-plasminogen. Binding was inhibited by lysine. $^{125}$I-albumin binding to liver slices was minimal and not inhibited by lysine. Activation of plasminogen by tissue plasminogen activator (t-PA) was enhanced by hepatocytes in primary culture. When lysine was included in the media, the enhanced rate of activation was no longer observed. After activation with t-PA, much of the plasmin remained associated with hepatocyte surfaces and was partially protected from inhibition by $\alpha_2$-antiplasmin. These studies suggest that hepatocyte plasminogen binding sites may provide important surface anticoagulant activity.

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Endothelial cells express highly specialized membrane macromolecules that function in hemostasis. Some of these macromolecules support coagulation, however, the primary activities are probably anticoagulant. Heparin-like proteoglycans on endothelial cell surfaces bind and activate antithrombin III, a potent inhibitor of intrinsic and common pathway proteinases. Thrombomodulin serves as a cofactor for the activation of the anticoagulant, protein C, by thrombin. Finally, tissue plasminogen activator (t-PA) binds specifically to the surfaces of endothelium.

Plasmin is the primary proteinase responsible for fibrin clot digestion. In the circulation, plasmin activation is most frequently catalyzed by t-PA. Recently, Hajjar et al. described an endothelial cell surface receptor that specifically binds plasminogen. t-PA activated receptor-bound plasminogen more readily than plasminogen in solution, suggesting a role for the plasminogen receptor as a cell surface anticoagulant.

Cell surface plasminogen binding sites have been demonstrated on cells other than endothelium, including circulating white cells, U937 promyeloid leukemia cells, a fetal lung fibroblast cell line, and platelets. However, plasminogen binding is not a ubiquitous property of cells in culture because some cell lines fail to bind significant quantities of the protein.

Like the endothelial cell, hepatocytes are directly exposed to plasma proteins and the highly specialized plasma membranes of these cells demonstrate many activities that are important in hemostasis. Hepatocyte receptors mediate the binding and endocytosis of proteinase inhibitor-proteinase complexes that form as a result of coagulation and fibrinolysis. Other receptors play an important role in the rapid catabolism of circulating t-PA.

This investigation was undertaken to determine if hepatocytes express plasminogen binding sites. Such binding sites might provide necessary membrane-associated anticoagulant activity because hepatocytes are in direct contact with plasma proteins. Results obtained from studies of primary cultures indicate that hepatocytes readily bind plasminogen. The boundzymogen is activated by t-PA at an enhanced rate despite the previously described inability of hepatocytes to rapidly catabolize the activator. Plasmin generated on the surfaces of hepatocytes demonstrates relative resistance to inhibition by $\alpha_2$-antiplasmin ($\alpha_2$AP). Finally, experiments performed with thin slices of intact liver demonstrate that hepatic cells as a group (ie, hepatocytes, Kupffer cells, and hepatic endothelium) manifest specific plasminogen binding activity without exposure to cell culture conditions.

Materials and Methods

Materials. Minimum essential medium with Earle's salts (MEM), Leibovitz L-15 medium, Earle's balanced salts (EBSS), and bovine crystalline insulin were purchased from GIBCO Laboratories (St. Louis, MA). H-D-Val-L-Leu-L-Lys-p-nitroanilide HCl (S-2251) was from Helena Laboratories (Beaumont, TX). Para-nitrophenylphosphate, type IV collagenase (clostridium), fetal calf serum, acid soluble rat tail collagen, and bovine serum albumin (BSA, 98% to 99% pure, fatty acid free) were from Sigma Chemical (St Louis). Na$^{125}$I was from Amershams (Arlington Heights, IL) and iodobeads were from Pierce Chemical (Rockford, IL).

Proteins. [Glu$^+$]Plasminogen was purified from human plasma according to the procedure of Deutsch and Mertz except that aprotinin (FBA Pharmaceuticals, New York) was added before chromatography (50 KIU/mL plasma). The three separate plasminogen preparations studied in this investigation contained mixtures of carbohydrate variants I and II. The carbohydrate variant II was present in slight excess (50% to 70%) as determined by acid gel electrophoresis.

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Wiman. Rat fibrinogen was purified using the ethanol fractionation procedure of Doolittle et al. The starting material was rat plasma that was passed through a lysine-Sepharose column to remove the plasminogen. Recombinant two chain t-PA was supplied by Dr S.V. Pizzo (Duke University). Goat antirat-fibrinogen antibody was purchased from Cappel (West Chester, PA) and antigoat immunoglobulin (IgG) acid phosphatase conjugated antibody was from Sigma. Bovine thrombin was also from Sigma.

Radiiodination. Plasminogen was radiiodinated using Iodo-beads as described by the manufacturer. Desalting was performed on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). Specific activities varied from 0.4 to 1.0 μCi/μg. Radioiodination did not result in plasminogen activation as determined by substrate assay using 0.4 μmol/L 125I-plasminogen and 0.8 mmol/L S-2251. Rat fibrinogen and albumin were radiiodinated similarly. The specific activities were 1.2 and 1.4 μCi/μg, respectively.

Isolation and culture of parenchymal hepatocytes. Hepatocytes were isolated from female Sprague-Dawley rats (100 to 150 g) using the two-step collagenase perfusion technique described by others. All solutions were oxygenated before perfusion. Isolated hepatocytes were washed three times with perfusion buffer and pelleted by centrifugation at 50 g for two minutes (4°C). The final pellet was suspended in MEM supplemented with 1.0 mmol/L pyruvate, 0.5 mmol/L aspartate, 0.2 mmol/L serine, 26 mmol/L NaCO3, 0.1 μmol/L insulin, 50 μg/mL gentamycin, 5% fetal calf serum, 10 μmol/L aprotinin, and 10 μg/mL leupeptin (plating medium). The cells were cultured in 35 mm collagen coated plates. (15 to 40 μg collagen per well as determined by the method of Peterson) and incubated at 37°C in 7% CO₂ for two to three hours. The medium was then exchanged and experiments were immediately performed. Hepatocytes prepared in this manner demonstrated >90% viability as judged by trypan blue exclusion. Contamination of these preparations by liver cells other than hepatocytes is generally <2%. Figure 1 shows a representative photomicrograph of the final hepatocyte preparation fixed with buffered formalin in the cell culture dish after two hours of incubation.

Plasminogen binding experiments. Radioiodinated plasminogen was incubated with cells at 37°C or at 4°C. The medium used at 37°C was the same as plating medium except that BSA (10 mg/mL) was substituted for the fetal calf serum. Studies at 4°C were performed in L-15 supplemented similarly except for the addition of 10 mmol/L HEPES. After binding, the medium was removed and the plates were washed three times with EBSS, 10 mmol/L HEPES, pH 7.4. Lysis solution (0.1 mol/L NaOH, 2.5% sodium dodecyl sulfate [SDS]) was added to each well and incubated overnight. Total radioactivity was determined in a γ counter (counting efficiency >75%). Protein content was determined by the method of Peterson.

In one set of experiments, different concentrations of L-lysine, ε-aminocaproic acid (EACA), or nonradiolabeled plasminogen were included with the 125I-plasminogen and the effect on radioligand binding was studied. In all other experiments, binding was studied in the presence or absence of 20 mmol/L lysine. Specific binding was defined as the difference between total binding (no lysine) and binding observed in the presence of lysine. In all reported studies, specific binding represented 80% to 90% of total binding. Control experiments were always performed in which the 125I-plasminogen was incubated in collagen-coated plates without cells. Some specific binding of plasminogen to the collagen was observed; however, this binding was consistently <20% of that determined in the presence of cells. Each cell binding measurement was corrected by subtracting the specific binding determined for collagen alone. Because only 20% to 40% of the surface collagen was exposed when cells were present, the final binding values presented here may be underestimated by approximately 10%.

The MEM and L-15 media used in this investigation contained 0.4 mmol/L lysine. In order to determine the effect of this lysine on the plasminogen binding data, experiments were performed at 4°C using EBSS containing 10 mg/mL BSA and 10 mmol/L HEPES, pH 7.4 instead of L-15.

Hepatocytes in suspension were counted using a hemocytometer. Protein and DNA contents were determined using the same cell preparations. The number of suspended cells per microgram of cell protein was 340 ± 60. The number of cells per nanogram of DNA was 57 ± 8. In the binding studies, protein content was used to standardize ligand binding data since the protein assay was more sensitive than the DNA assay. After two hours in culture, the DNA-to-protein ratio decreased by 20% to 30%. This decrease may reflect subcellular matrix deposition; a possible source of error in our calculations of cell number. This small possible error would only be reflected in receptor number determinations. No additional change in DNA-to-protein ratio was observed after 7.0 hours in culture.

All hepatocyte binding experiments were performed at least three times with duplicate or triplicate determinations for each data point. Saturation isotherms were fitted to the equation for a rectangular hyperbola (Y = [A · X]/[B + X]) where B = Kₐ using the nonlinear regression method of Marquardt. The same data were plotted using the Scatchard transformation. The Kₐ and cellular binding capacity were then determined by linear regression.

Uptake and degradation experiments. 125I-plasminogen was incubated with hepatocytes at 4°C or 37°C as described above except that the aprotinin and leupeptin were omitted from all solutions. At various times, the supernatants were removed and treated with trichloroacetic acid (TCA) (final concentration 8.0% wt/vol). Soluble and precipitable radioactivity was measured. Of the initial 125I-plasminogen, 98.4 ± 0.4% was precipitable. 125I-plasminogen that remained associated with the cells after washing was subjected to SDS-polyacrylamide gel electrophoresis using the pH 7.4 buffer system of McElwan. Reducing and nonreducing conditions were studied. The plasminogen in these gels was analyzed by autoradiography. In some experiments, the supernatants were studied by electrophoresis and autoradiography as well.

Determination of secreted fibrinogen. An enzyme-linked immunosorbent assay (ELISA) was developed to study fibrinogen synthe-

Fig 1. The typical appearance of the monomorphic primary hepatocyte culture two hours after attachment to the collagen-coated wells. Phase contrast original magnification × 325.
sis and secretion by hepatocytes in culture. A standard curve was generated using various concentrations of \(^{125}\)I-rat fibrinogen. The fibrinogen was allowed to adsorb in 35 mm culture cell wells for two hours. BSA (10 mg/mL) was then added to each well for 20 minutes followed by extensive washing. The primary antibody, diluted 1:500, was incubated with the adsorbed fibrinogen for 45 minutes at 23\(^\circ\)C. After washing, secondary antibody (1:1,000) was added for 45 minutes. p-Nitrophenylphosphate (1.0 mg/mL) was used to detect secondary antibody. The adsorbed \(^{125}\)I-fibrinogen was then dissociated with 0.1 mol/L NaOH, 2.5% SDS, and quantitated in a \(\gamma\) counter. A plot of ELISA results vs adsorbed fibrinogen was linear (correlation coefficient 0.99). In some experiments, the adsorbed fibrinogen was treated with thrombin (7 U/mL) for five minutes at 37\(^\circ\)C. No change in ELISA results was detected.

Hepatocytes were plated for two hours and then transferred into the media used in the \(^{125}\)I-plasminogen binding experiments. At various times, the plates were washed with EBSS, 10 mmol/L HEPES, pH 7.4. The amount of fibrinogen/fibrin associated with the cells and collagen matrix was determined by ELISA using the procedure described above.

**Plasminogen binding to thin liver slices.** Thin liver slices were prepared from rat livers perfused in situ with 10 mmol/L HEPES, 142 mM NaCl, 7.6 mmol/L KCl, pH 7.4 at 4\(^\circ\)C. Core sections from the center of the liver (3 x 5 mm) were sliced into 500 \(\mu\)m uniform sections using a McIlwain tissue chopper. These slices were allowed to equilibrate at 4\(^\circ\)C for ten minutes in L-15 supplemented as described for the cell culture experiments. Each slice was then transferred into medium that included \(^{125}\)I-plasminogen or \(^{125}\)I-albumin with or without 20 mmol/L lysine. At various times, the slices were removed from the medium and washed twice with EBSS/HEPES buffer. After determining the amount of bound radioactivity in a \(\gamma\) counter, the slices were totally dissolved by incubation with NaOH and SDS at 37\(^\circ\)C for 18 hours. Protein assays then were performed.

**Activation of plasminogen by t-PA.** Hepatocytes obtained by collagenase perfusion were plated in MEM supplemented as described above except that the aprotonin and leupeptin were omitted. After two hours, the plates were washed thoroughly with EBSS, 10 mmol/L HEPES, 1.0 mg/mL BSA, pH 7.4. Empty plates and plates that contained only collagen were washed with the same solution. Plasminogen (30 \(\mu\)g/mL), S-2251 (0.5 mmol/L) and various concentrations of t-PA were added to the wells. In some experiments, 20 mmol/L lysine was also added. The plates were then incubated at 37\(^\circ\)C for 95 minutes. Substrate hydrolysis was stopped with 0.1 mL glacial acetic acid. Absorbances were determined at 406 nm.

**Inhibition of plasmin by \(\alpha\)-AP.** Plasminogen (30 \(\mu\)g) and t-PA (2 U) were added to cultures of washed hepatocytes in EBSS, 10 mmol/L HEPES, 1.0 mg/mL BSA, pH 7.4 (volume, 0.8 mL). After 90 minutes, the medium was separated from the cells and saved (solution 1). Fresh medium (no additional t-PA or plasminogen) was immediately added to the remaining cells (solution 2). In some wells, the medium and cells were not separated after the initial 90-minute incubation (solution 3). \(\alpha\)-AP and S-2251 (0.5 mmol/L), or just S-2251 alone, were added simultaneously to all three solutions. The final concentration of \(\alpha\)-AP was 0.4 \(\mu\)mol/L. Since only a small fraction of the plasminogen was activated, the molar ratio of \(\alpha\)-AP to plasm in the final solutions was >10. Plasmin activity was determined based on S-2251 hydrolysis (ten minutes). Equivalent experiments were performed in cell culture plates without cells. Some of the wells were coated with collagen.

In order to demonstrate that \(\alpha\)-AP is completely active in the presence of hepatocytes, the proteinase inhibitor (40 mmol/L) was incubated with cells in EBSS/HEPES/BSA for 15 minutes. S-2251 and different concentrations of plasmin (activated separately with urokinase) were then added sequentially to the wells. The same experiment was performed in wells that did not have hepatocytes. Plasmin activity was determined based on substrate hydrolysis.

**RESULTS**

**Plasminogen binding to hepatocytes in culture.** \(^{125}\)I-Plasminogen (0.1 \(\mu\)mol/L) bound readily to hepatocytes in culture at 4\(^\circ\)C and at 37\(^\circ\)C. When 20 mmol/L lysine was included in the media, 80% to 90% of the binding was eliminated. The change in specific binding as a function of time is shown in Fig 2. At 4\(^\circ\)C, near maximum binding was achieved within two hours. At 37\(^\circ\)C, maximum plasminogen binding was achieved sooner; however, the absolute quantity of bound radioligand was not significantly different.

Hepatocytes in primary culture synthesize and secrete plasminogen, which could affect the results of the 37\(^\circ\)C binding experiments. Based on published values, accumulation of new plasminogen in the medium after 5.0 hours was well under 100 ng/mL. In this laboratory, a substrate assay that detects 50 ng/mL of plasminogen in cell culture medium was developed (Braud and Gonias, unpublished data); however, we were unable to detect plasminogen synthesis at 5.0 hours, consistent with the previous investigation. Because over 9.0 \(\mu\)g of \(^{125}\)I-plasminogen was added to each well in the binding experiments presented above, the data were most likely not affected by synthesized plasminogen.

The effect of lysine on plasminogen binding to the hepatocytes was concentration dependent. The concentration required for 50% inhibition was 1.0 mmol/L. The lysine present in the L-15 and MEM media (0.4 \(\mu\)mol/L) reduced specific binding by approximately 30%.

Reversibility of plasminogen binding to hepatocytes was studied by radioligand displacement at 4\(^\circ\)C. \(^{125}\)I-Plasminogen (0.1 \(\mu\)mol/L) was incubated with hepatocytes for 45 minutes. Lysine (10 mmol/L), EAACA (10 mM), or nonradiolabeled plasminogen (6.0 \(\mu\)mol/L) was then added to the media. Radioligand binding was compared one hour later (Fig 3). In each case, \(^{125}\)I-plasminogen dissociated from the cells. Residual binding after addition of lysine was equivalent to the level

![Graph](https://via.placeholder.com/150)
detected when lysine and radiolabeled plasminogen were added at the same time. The 60-fold molar excess of plasminogen displaced over 70% of the radioligand. Lysine and EACA displace ^125I-plasminogen by binding to the radioligand; however, the nonradiolabeled plasminogen most likely competed directly for cell surface binding sites. These results demonstrate that plasminogen binding to hepatocytes is reversible.

^125I-Plasminogen binding to hepatocytes was studied as a function of plasminogen concentration at 4°C. Figure 4 shows the results of three separate experiments. When the plasminogen concentration was >2.0 μmol/L, specific binding of radioligand to the collagen was significant (>20% of the binding observed with cells), suggesting that the collagen contains a low-affinity high-capacity plasminogen binding site. This collagen site precluded hepatocyte binding experiments with the concentrations of plasminogen (2.0 to 15 μmol/L) required to incontrovertibly demonstrate saturation. Within the plasminogen concentration range studied, the percentage of total radioligand bound to the cells was <0.6%. The IC₅₀ for binding of ^125I-plasminogen to hepatocytes was 0.59 ± 0.16 μmol/L, as determined from the saturation isotherm by nonlinear regression (r² = 0.99) and the Scatchard transformation by linear regression (r² = 0.93). The number of sites per cell was 14.1 ± 1.1 x 10⁶. These values are in agreement with previously published constants, suggesting that the hepatocyte plasminogen receptor may be equivalent to sites on other cells.

**Fibrinogen secretion.** Hepatocytes in primary culture synthesize and secrete proteins that might bind plasminogen, the most prominent of which is fibrinogen. An ELISA was formulated to study the effect of fibrinogen secretion on the plasminogen binding data (Fig 5). This ELISA detected only

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**Fig 3.** Reversibility of ^125I-plasminogen binding at 4°C after addition of lysine (10 mmol/L), EACA (10 mmol/L), or nonradiolabeled plasminogen (6 μmol/L). Rat hepatocytes in primary culture were incubated with ^125I-plasminogen at 4°C in EBSS, 10 mmol/L HEPES, 10 mg/mL BSA, pH 7.4. After 45 minutes, the competing ligands were added. Incubation continued for one additional hour. ^125I-Plasminogen binding and cell protein were determined as described in the text. The bar graphs labeled 45 minutes and 105 minutes show binding in wells that received no competing ligand.

**Fig 4.** Concentration dependence of binding of ^125I-plasminogen to hepatocytes in primary culture. Incubations were conducted at 4°C for four hours. Specific binding is plotted as a function of ligand concentration in panel A. The error bars represent one standard deviation. The same data are plotted according to the method of Scatchard in panel B.

**Fig 5.** Secretion of fibrinogen by rat hepatocytes in primary culture at 37°C (C) and 4°C (D) as determined by ELISA. Studies were begun after an initial two-hour plating period at 37°C (0 hour). Wells were washed extensively before performing the assay. Therefore, only the fibrinogen associated with the cells or collagen was detected. The average collagen value was determined from collagen controls run with each time course. The incubation media were MEM, BSA (37°C), and L-15, BSA (4°C).
fibrinogen or fibrin associated with the cells or collagen because the medium was removed and the wells were washed extensively before the assays were performed. Some fibrinogen was detected in the primary hepatocyte cultures after the initial two-hour plating period. Evidence for continued fibrinogen secretion was obtained at 37°C; however, no additional fibrinogen was detected when the cells were incubated at 4°C. Approximately 5 ng of fibrinogen was detected in wells that contained only collagen. The amount of fibrinogen and fibrin detected in hepatocyte cultures at 4°C was always <5 ng. In the experiments described above, specific plasminogen binding to the acellular collagen coated plates was only 3% to 20% of that observed with the plated hepatocytes (85 femtomoles compared to 1,271 fmol with 0.4 μmol/L 125I-plasminogen in a representative experiment). Therefore, the quantity of secreted fibrinogen was insufficient to account for the vast majority of the plasminogen binding to hepatocytes.

Plasminogen degradation in culture. The binding experiments presented above suggest that plasminogen is not altered when bound to the hepatocyte membrane at 4°C. 125I-Plasminogen was incubated with hepatocytes for five hours at 37°C. During this time period, there was no significant increase in TCA soluble radioactivity. Using similar preparations of rat hepatocytes, others have demonstrated degradation of 125I-1-PA into TCA-soluble products at a rate of approximately 5% to 10%/h.14,16

SDS-gel electrophoresis under reducing conditions was performed to detect plasminogen activation or degradation that might occur during incubation with hepatocytes. Plasminogen activation involves conversion of the single-chain glycoprotein (Mr ~ 90,000) into two major species (Mr ~ 60,000 and Mr ~ 25,000).28 In these experiments, 125I-plasminogen was incubated with the cells for up to five hours at 37°C. Cell-associated radioactivity was separated from the media and both were analyzed by electrophoresis. As shown in Fig 6, no evidence of plasminogen activation or degradation was detected in either phase. There was also no evidence for conversion of [Glu]-plasminogen to [Lys]-plasminogen; however, the gel system had only limited sensitivity to this transition because mixtures of the two carbohydrate variants were studied.

Plasminogen binding to thin slices of liver. Thin sections of whole rat liver were isolated and prepared at 4°C so that significant protein degradation or synthesis could not occur. The procedure does not involve the use of collagenase or other proteinases that might alter the cell membranes. Figure 7 shows that 125I-plasminogen bound to the thin liver slices at 4°C and that >60% of the binding was inhibited by 20 mmol/L lysine. The change in specific binding with time was similar to that observed with the hepatocyte monolayer cultures. After incubation for three hours with 0.1 μmol/L 125I-plasminogen, approximately 1.0 × 10⁶ molecules of radioligand specifically bound to each microgram of liver slice protein. The plasminogen binding capacity of the individual cell in the liver slice cannot be determined because organ connective tissue contributes significantly to the total protein content of the tissue; however, when specific binding to the hepatocyte monolayers and tissue slices was expressed in comparable units (fmol/μg protein), the level of binding was similar.

In control experiments, the binding of 125I-albumin to thin liver slices was studied. The concentration of radioligand and the incubation conditions were unchanged. Total binding of 125I-albumin after five hours of incubation was <15% of that observed with plasminogen. When 20 mmol/L lysine was included in the incubation media, the level of albumin binding remained the same. These results demonstrate that the effect of lysine on plasminogen binding to liver slices is specific.
Plasminogen activation. Binding of plasminogen and t-PA to endothelial cell surfaces results in more efficient activation of the zymogen. The problem of plasminogen enhancement of the rate of plasminogen activation by t-PA was complicated because the cells rapidly catabolized the activator. Figure 8 shows that the primary hepatocyte cultures enhanced the rate of plasminogen activation by t-PA. A similar effect was not observed with the collagen-coated wells.

Plasminogen activation by t-PA was studied in the presence of 20 mmol/L lysine. In the absence of cells, lysine enhanced the rate of activation, consistent with the work of Urano et al. These investigators demonstrated that EACA binds [Glu']-plasminogen, converting the zymogen into a better substrate for t-PA. In the presence of hepatocytes, lysine strongly antagonized the plasminogen activation reaction. Plasmin was generated at extremely low levels, even in comparison with the control reactions performed in the absence of cells. In these experiments, the lysine most likely inhibited the cell surface interaction responsible for the enhanced rate of plasminogen activation. The most plausible site for the lysine effect is the plasminogen receptor; however, we cannot rule out the possibility that lysine interfered with critical t-PA surface interactions as well. Once the surface-enhancing effect was eliminated by lysine, cellular catabolism of t-PA was apparent in the data.

Plasmin inhibition. The most important inhibitor of plasmin in the circulation is α2AP. In order to demonstrate that α2AP is active in the presence of hepatocytes, a standard concentration of inhibitor (40 nmol/L) was incubated with cells for 15 minutes. Different concentrations of plasmin (30 mmol/L to 240 nmol/L) and 0.5 mmol/L S-2251 were then added to each well. α2AP inhibited plasmin and this was observed as a decrease in the rate of substrate hydrolysis. Equivalent reductions in substrate hydrolysis were observed when the same experiment was performed in wells that did not have hepatocytes (data not shown). Therefore, under the experimental conditions studied here, hepatocytes did not affect the activity of α2AP.

In the next series of experiments, plasminogen was activated in the presence and absence of hepatocytes. Plasmin regulation by α2AP was studied by adding S-2251 alone or α2AP and S-2251 simultaneously to each well (Fig 9). When no cells were present, plasmin inhibition by α2AP was essentially immediate since substrate hydrolysis in the presence of inhibitor was reduced by over 95%. In the presence of cells, significantly more substrate hydrolysis occurred (>20% of control), suggesting that at least a fraction of the plasmin was partially protected from inhibitor.

In order to further examine this effect, plasminogen was activated in the presence of hepatocytes as described above. After 90 minutes, the media were separated from the cells. Approximately 95% of the plasmin activity was recovered and the majority of this activity remained associated with the hepatocytes (Fig 9). Plasmin inhibition experiments were performed using the two separated fractions. α2AP inhibited the plasmin in the aspirated media efficiently when the inhibitor and S-2251 were added simultaneously. Substrate hydrolysis approximated that observed when plasminogen
was activated in acellular wells (<5% of control). By contrast, α2AP functioned poorly as an inhibitor of plasminogen in the reconstituted wells of hepatocytes. Substrate hydrolysis remained near 35% of that demonstrated in the absence of α2AP. These data strongly suggest that receptor-bound plasmin is less reactive with α2AP.

**DISCUSSION**

Recent investigations, performed mainly with endothelial cells, suggest that the cell surface is potentially thrombogenic. This potential is counteracted by anticoagulant and profibrinolytic activities that are also associated with cell membranes. Plasminogen receptors on endothelium and WBCs may provide important profibrinolytic activity. The enhanced rate of plasminogen activation by t-PA on the surfaces of endothelium supports this hypothesis. Hepatocyte surface membranes are directly exposed to the plasma as it circulates through the sinusoidal spaces. This exposure occurs primarily because hepatic endothelial cells have many fenestrations and fail to develop intercellular contacts characteristic of extrahepatic endothelium. This microanatomy suggests that hepatocytes should require anticoagulant membrane activity. The data presented here demonstrate that hepatocytes in primary culture bind plasminogen readily when the protein is present at concentrations similar to that present in plasma. Plasminogen binding is reversible and does not appear to result in significant ligand internalization. The number of binding sites and the Kd are similar to the values reported for endothelium. In addition, plasminogen that is bound to hepatocyte membranes is more readily activated by t-PA and less readily inactivated by α2AP. These data suggest that plasminogen binding by hepatocytes may be important in maintaining plasma fluidity in the sinusoidal spaces.

Binding experiments were performed with thin liver slices to complement the hepatocyte primary culture studies. The average thickness of the slice was eight cells. The major advantages of this system are: the slices are isolated without exposure to extrinsic proteinases such as collagenase; the slices are maintained at 4°C throughout the procedure, thereby preventing synthesis and secretion of proteins such as fibrinogen and fibronectin; hepatic architecture is preserved in the in vivo configuration. Of course, the major disadvantage is that all liver cells are present together and the activities of each cannot be differentiated. Specific binding of plasminogen to the slices was observed. Interestingly, the amount of specific plasminogen binding per microgram of cell protein was approximately the same for the slices and for hepatocytes in primary culture. These experiments demonstrate that liver cells as a group manifest specific plasminogen binding activity without exposure to a cell culture environment. In addition, these studies support the body of literature concerned with lysine-displaceable plasminogen binding to surfaces by suggesting that this binding occurs in vivo. By contrast, Hatton et al recently studied plasminogen binding to intact and deendothelialized rabbit aorta in vivo and in vitro. These investigators were unable to demonstrate displacement of bound plasminogen by e-aminocaproic acid. In view of our liver slice data, the reason for this negative result in the in vitro system is unclear.

In the circulation, t-PA is the primary activator of plasminogen; however, this reaction is inefficient unless other factors are present. Perhaps the most important cofactor is fibrin, which decreases the Km for activation of plasminogen by t-PA by a factor of 103. Other soluble proteins such as thrombospordin and histidine-rich glycoprotein may enhance plasminogen activation by t-PA as well. In each case, plasminogen and t-PA bind to the cofactor. By analogy, association of both plasminogen and t-PA with cell surfaces may be critical for enhancing the rate of zymogen activation. The data presented here suggest that t-PA may be active on the hepatocyte plasma membrane. Specific hepatocyte surface binding sites for t-PA have been described; however, occupancy of these sites supposedly results in rapid ligand catabolism. In our activity assays, when the surface interaction responsible for the increased rate of plasminogen activation was inhibited with lysine, evidence for t-PA catabolism by hepatocytes was observed. Further investigation will be required to determine if the t-PA binding sites that result in catabolism and the sites that mediate plasminogen activation are the same. Interestingly, when plasminogen was incubated with hepatocytes in the absence of t-PA, activation was not observed (Fig 8). This suggests that no intrinsic rat plasminogen activators were retained by the hepatocytes during the isolation and plating procedure.

α2AP is the primary inhibitor of circulating plasmin; however, other inhibitors such as α2-macroglobulin also react rapidly with this proteinase. Plasmin that was generated in the presence of hepatocytes remained largely associated with the cell surfaces. This pool of plasmin was less reactive with α2AP. A similar result was reported for GM1380 cells by Plow et al. These investigators used electrophoresis and autoradiography to study the reaction of receptor-bound plasmin with α2AP. Due to the difference in technique, their data and the results reported here cannot be directly compared; however, the reduction in reaction with α2AP caused by the GM1380 cells was profound. We propose that the plasminogen receptor system should provide an excellent opportunity to study regulation of membrane associated proteinases by the plasma proteinase inhibitors in general.

**REFERENCES**

4. Esmon CT: Protein C. Prog Hemost Thromb 7:25, 1984
5. Hajjar KA, Hamel NM, Harpel PC, Nachman RL: Binding of...
27. Bohmfalk JF, Fuller GM: Plasminogen is synthesized by primary cultures of rat hepatocytes Science 209:408, 1980
Plasminogen binding to rat hepatocytes in primary culture and to thin slices of rat liver

SL Gonias, LL Braud, WA Geary and SR VandenBerg