

The Pathogenesis of Accelerated Fibrinolysis in Liver Cirrhosis: A Critical Role for Tissue Plasminogen Activator Inhibitor

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The pathogenesis of accelerated fibrinolysis in liver cirrhosis was investigated by comparing the results of specific assays for tissue plasminogen activator (tpa) antigen, tpa activity, tpa inhibitor, and alpha-2 plasmin inhibitor (a2PI) in 12 patients with cirrhosis and markedly accelerated fibrinolysis (dilute whole blood clot lysis time (DWBCLT) < two hours), in nine patients with cirrhosis and moderately accelerated fibrinolysis (DWBCLT two to four hours), and in nine patients with cirrhosis and normal fibrinolysis (DWBCLT > four hours). Mean tpa antigen was markedly increased in all three groups, but no correlation was observed between overall fibrinolytic activity as measured

by the DWBCLT and the level of tpa antigen. In contrast, there was a significant correlation between overall fibrinolytic activity and tpa activity and an equally significant correlation between fibrinolytic activity and decreased tpa inhibition. Mean a2PI activity was significantly lower than normal in groups 1 and 2 but was normal in group 3. The pathogenesis of accelerated fibrinolysis in liver cirrhosis thus appears to depend critically on the capacity of plasma inhibitors to inhibit increased circulating tpa antigen. Reduced a2PI also appears to play a role.

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ACCCELERATED fibrinolysis is a recognized complication of cirrhosis.¹⁻³ The liver is not only an important site of clearance of circulating plasminogen activators⁴ but also synthesizes the main plasmin inhibitor, alpha-2 plasmin inhibitor (a2PI).⁵ Thus a multifactorial pathogenesis of accelerated fibrinolysis in cirrhosis has been presumed, with contributions from elevated levels of circulating plasminogen activators caused by decreased clearance^{6,7} and reduced inhibition of plasmin due to diminished a2PI synthesis.⁸ Previously published studies of fibrinolysis in cirrhosis have not, however, investigated the interrelationship of multiple abnormalities of the fibrinolytic system, nor have they attempted to correlate these abnormalities with either overall fibrinolytic activity of blood or with clinical manifestations. Moreover, the recent discovery of one or more rapid inhibitors of tissue-type plasminogen activator (tpa) in plasma⁹⁻¹¹ has revealed a new complexity in the physiologic regulation of fibrinolysis. The behavior of tpa inhibitor in liver cirrhosis has yet to be investigated.

We previously reported that accelerated fibrinolysis may have significant clinical consequences for individuals with liver cirrhosis.¹² In comparing bleeding histories of 46 patients with markedly accelerated fibrinolysis (DWBCLT < two hours) to 44 patients with normal fibrinolysis (DWBCLT > four hours), we found that patients with accelerated fibrinolysis were at increased risk for major soft-tissue hemorrhage after trauma and exhibited a trend toward increased intracranial bleeding, usually fatal.¹² Other hemostatic abnormalities may have contributed to the increased bleeding in patients with accelerated fibrinolysis, however, since this group had a significantly longer mean partial thromboplastin time (PTT) and higher mean levels of fibrin/fibrinogen degradation products (fdp/FDP) than the group with normal fibrinolysis. Nevertheless, in several patients with severe bleeding, accelerated fibrinolysis was the only marked hemostatic abnormality present.

This study raised many important questions. What specific abnormalities of fibrinolysis contribute to accelerated fibrinolysis in patients with cirrhosis? Which are the most important? Why does fibrinolytic activity vary so greatly among individuals with apparently equally severe liver disease? In an attempt to answer these questions, we undertook a comprehensive evaluation of the fibrinolytic system in patients with liver cirrhosis using newly developed, highly

specific assays for tpa antigen and activity, tpa inhibitor, and a2PI.

MATERIALS AND METHODS

Patient selection. The records of the Coagulation Laboratory of the Division of Hematology at Los Angeles County (LAC) University of Southern California (USC) Medical Center were reviewed to identify those patients with liver disease in whom fibrinolytic activity had been measured with the DWBCLT. The diagnosis of cirrhosis was confirmed by a review of the medical records for each patient identified. Patients were considered to have cirrhosis if they showed either biopsy evidence of cirrhosis or documented portal hypertension (presence of esophageal varices or redistribution of tracer to spleen and bone marrow on liver-spleen scanning) in the setting of parenchymal liver disease and appropriate historic background (heavy alcohol consumption or chronic hepatitis). Patients with acute hepatic necrosis secondary to viral or toxic agents were excluded. All patients meeting these criteria were included in the analysis, provided sufficient stored plasma obtained at the same time as the DWBCLT was available for performance of all the fibrinolytic assays. To determine the possible influence of transfused blood products on the results of fibrinolytic assays, the records of the LAC-USC Medical Center blood bank were reviewed, and notation was made of the quantity and type of all blood products received in the seven days preceding the date of plasma sampling. In calculating the total amount of plasma received by each patient, we assumed that one unit of fresh frozen plasma contained 225 mL of plasma, one unit of packed red cells 75 mL, and one unit of platelets 50 mL. For determination of the normal range for fibrinolytic assays, blood

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was obtained from resting healthy adult donors without history of bleeding or thrombosis. The principles of the Declaration of Helsinki were followed in the care of all patients.

Preparation of plasma samples and euglobulin precipitates. All samples were obtained by atraumatic venipuncture and anticoagulated with one-tenth volume of balanced citrate. Plasma was prepared by centrifugation for ten minutes at 10,000 rpm at 4°C and stored at -20°C or -85°C until assayed. The euglobulin fraction of plasma was obtained by diluting 0.5 mL of plasma in 20 volumes of ice-cold 0.014% acetic acid (pH 5.6) prepared fresh daily from a 1.4% stock solution. After incubating for at least 30 minutes on melting ice, the precipitate was recovered by centrifugation for ten minutes at 2,500 rpm at 4°C and resuspended in 0.5 mL of 0.01 mol/L phosphate, 0.15 mol/L NaCl, pH 7.4, containing 0.1% (vol/vol) Tween 80 (PBS-Tween).

Materials. Tissue plasminogen activator (tpa) was purified from the conditioned medium of Bowes human melanoma cell cultures as previously described.¹³ Human plasminogen (glu/lys mixture) and goat antihuman tpa antibody were obtained from American Diagnostica (Greenwich, CT). Cyanogen bromide-fragmented fibrinogen was prepared as previously described.¹⁴ D-val-leu-lys-pNA (S-2251) was obtained from Helena Laboratories (Beaumont, TX). Crystallized bovine serum albumin was obtained from Sigma Chemical (St. Louis). All other materials were of the highest quality obtainable.

Measurement of tpa antigen. The ELISA method of Bergsdorf et al,¹⁵ commercially available through American Diagnostica (Greenwich, CT), was used. Briefly, 96-well flat-bottomed microtiter plates (Nunc Immunoplate I-F) were coated overnight at room temperature with goat antihuman tpa antibody, followed by washing with 0.15 mol/L NaCl containing 0.1% (vol/vol) Tween 80 (saline-Tween), and blocking of unreacted binding sites with 2% bovine serum albumin (BSA) for one to two hours at room temperature. After washing again, diluted euglobulin precipitates of test plasmas and a range of concentrations (0.05 to 1.6 ng/mL final in the wells) of purified tpa standard (as supplied by the manufacturer) were added and allowed to incubate overnight at room temperature. After additional washing, goat antihuman tpa antibody conjugated to peroxidase was added and allowed to incubate for two hours at room temperature. Then the plate was washed again, and ortho-phenylene diamine substrate was added. After 30 minutes in the dark at room temperature, the reaction was stopped by adding 4.5 mol/L H₂SO₄, and the absorbance at 492 nm was read on an eight-channel scanning spectrophotometer (Titertek Multiskan, Flow Laboratories, Irvine, CA). Each euglobulin precipitate was assayed in duplicate at four dilutions in PBS-Tween (fourfold, eightfold, 16-fold, and 32-fold), and the mean value of all dilutions falling on the linear portion of the standard curve was reported. Tpa antigen values in 25 healthy subjects were normally distributed, with a mean of 4.9 plus or minus 2.6 ng/mL (SD) and a range of 0.9 to 11.2 ng/mL. With this assay, quantitative recovery of a wide range of concentrations of purified tpa added to plasma (5 to 50 ng/mL final) was observed, indicating that both free tpa and the tpa/tpa inhibitor complex were detected with equal efficacy.

Measurement of tpa activity. A solid-phase bioimmunoassay similar to that described by Mahmoud and Gaffney¹⁶ was employed to measure tpa activity in euglobulin precipitates of plasma. Microtiter plates were coated overnight with goat antihuman tpa antibody followed by washing and blocking with 2% BSA as described above. Then euglobulin precipitates and a range of concentrations of purified tpa standard (as supplied for the tpa ELISA) were added and incubated for one to two hours at room temperature. After washing, purified human plasminogen (10 µg/mL final) and the plasmin substrate S-2251 (0.15 mmol/L final) in PBS-Tween were added, and color was allowed to develop overnight at 37°C. Absorb-

ance was read at 405 nm on the eight-channel spectrophotometer. Each euglobulin precipitate was assayed in duplicate at three dilutions (twofold, fourfold, and eightfold) and the mean value reported. With this assay tpa activity was not detectable in plasma from most resting normal subjects.

Measurement of tpa inhibitor. The assay of Juhan-Vague et al¹⁷ was used. Tissue plasminogen activator (tpa) purified from Bowes human melanoma cell cultures was added to prewarmed plasma at a final concentration of 50 ng/mL and allowed to incubate for exactly five minutes at 37°C, followed by acidification and recovery of the euglobulin precipitate as described above. The residual tpa activity was measured in microtiter plate wells, as described by Verheijen et al,¹⁴ using cyanogen bromide-fragmented fibrinogen as a stimulant. Since with this method the presence of free tpa activity in the test plasmas could result in a falsely low apparent tpa inhibitor level, each test plasma was also assayed without addition of purified tpa, and the difference in activity between the samples with and without added tpa was used to calculate the tpa inhibitor level. Tissue plasminogen activator (tpa) inhibitor was expressed as ng/mL of added tpa inhibited. Mean tpa inhibitor in 19 normal healthy subjects was 41.9 plus or minus 9.8 ng/mL.

Measurement of a2PI activity. The a2PI activity in plasma was measured spectrofluorometrically using the commercial "Protopath" assay of Dade (Miami), modified according to the method of Matsuda et al¹⁸ to eliminate any effect of alpha-2 macroglobulin on the assay. Briefly, plasma samples were incubated for five minutes at 37°C with Tris-saline buffer pH 7.5 containing 0.2 mol/L methylamine HCl to inhibit alpha-2 macroglobulin, followed by addition of plasmin, further incubation for exactly one minute at 37°C, and measurement of residual plasmin activity spectrofluorometrically. Results of test samples were expressed as a percentage of the activity in pooled normal plasma. The normal range for 15 healthy subjects was 73% to 119% (mean ± 2 SD).

Other assays. The DWBCLT was performed as previously described.¹² Briefly, 0.2 mL of freshly drawn blood was pipetted into 1.7 mL of ice-cold acetate-citrate buffer pH 7.4, inverted several times to mix, and kept on melting ice until 0.1 mL of a 50 U/mL thrombin solution could be added. Duplicate samples so processed were incubated at 37°C until lysis occurred. Quick prothrombin times and activated partial thromboplastin times were performed in standard fashion. Levels of fdp/FDP were determined using the Thrombo-Wellcotest (Burrhoughs-Wellcome, Research Triangle Park, NC).

Statistical methods. Significance of differences in mean laboratory parameters among the patient groups and normal subjects was assessed using the two-tailed Student's *t* test, with a *P* value of <0.05 considered to be significant. Correlation coefficients were calculated using simple linear regression.

RESULTS

Thirty patients, accrued between January 1983 and June 1986, had sufficient plasma available for performance of all fibrinolytic assays. Twelve patients (group 1) had markedly accelerated fibrinolysis (DWBCLT < two hours). Nine patients (group 2) had moderately accelerated fibrinolysis (DWBCLT between two and four hours). Nine patients (group 3) had normal fibrinolysis (DWBCLT > four hours). Results of standard coagulation assays and liver function tests are displayed in Table 1. Although group 1 patients had a lower mean serum albumin, higher mean total bilirubin, longer mean prothrombin time and PTT, and higher level of fdp/FDPs than groups 2 and 3, the only statistically significant difference among the three groups was observed for the

Table 1. Coagulation and Liver Function Tests (Mean $\pm$ SD)

	Group 1	Group 2	Group 3
Serum albumin (g/dL)	2.6 $\pm$ 0.5	2.7 $\pm$ 0.6	3.0 $\pm$ 0.6
Total bilirubin (mg/dL)	8.3 $\pm$ 6.4	4.4 $\pm$ 2.6	4.1 $\pm$ 4.7
Fibrinogen (mg/dL)	119 $\pm$ 51	214 $\pm$ 43	276 $\pm$ 118
Prothrombin time (seconds)	22.7 $\pm$ 5.2	18.7 $\pm$ 3.3	19.3 $\pm$ 5.4
PTT (seconds)	102.8 $\pm$ 49.9	74.9 $\pm$ 15.1	74.9 $\pm$ 41.3
fDP/FDP (μ g/mL)	22.0 $\pm$ 28.5	17.8 $\pm$ 25.7	17.8 $\pm$ 16.8
Plasma received (mL)	480 $\pm$ 690	344 $\pm$ 601	1661 $\pm$ 2937

fibrinogen level, which was significantly lower in groups 1 and 2 than in group 3.

Fibrinolytic data are summarized in Table 2. A similarly marked increase in mean tpa antigen was seen in all three groups when compared with the values obtained in normal subjects. Tpa antigen showed no correlation with overall fibrinolytic activity as measured by the DWBCLT ($r = -0.13$, $P > 0.20$). In contrast, tpa activity was significantly correlated with the DWBCLT ($r = -0.45$, $P < 0.05$) and differed markedly among the three groups, being markedly increased in group 1 and essentially undetectable in group 3. The tpa inhibitor also correlated significantly with the DWBCLT ($r = 0.43$, $P < 0.05$) and was significantly lower in group 1 than in group 3. The tpa inhibitor did not correlate significantly with serum albumin ($r = -0.09$, $P > 0.50$) but did correlate significantly with a2PI ($r = 0.47$, $P < 0.05$).

Mean a2PI activity was significantly lower than normal in groups 1 and 2 and was normal in group 3. The a2PI did not correlate significantly with the DWBCLT ($r = 0.35$, $P > 0.05$) or with serum albumin ($r = 0.32$, $P > 0.10$) but did correlate significantly with fibrinogen ($r = 0.59$, $P < 0.01$) and with tpa inhibitor ($r = 0.47$, $P < 0.05$).

Five patients in group 1, three patients in group 2, and five patients in group 3 received blood products prior to measurement of fibrinolytic parameters. The amount of plasma received did not differ significantly among the three groups and did not correlate significantly with any of the fibrinolytic parameters.

DISCUSSION

Tissue plasminogen activator (tpa) is synthesized by endothelium and cleared by the liver. Thus diminished hepatic

clearance in cirrhosis would be expected to produce elevated levels of circulating tpa. Using the ELISA technique developed by Bergsdorf et al,¹⁵ we found that marked increases in tpa antigen are common in patients with cirrhosis. Surprisingly, however, the level of tpa antigen did not correlate with overall fibrinolytic activity as measured by the DWBCLT. In contrast, the level of free tpa activity correlated strongly with overall fibrinolytic activity. We interpret this to indicate that in patients with cirrhosis the amount of tpa inhibitor available to bind and neutralize circulating tpa is a critical factor in the development of accelerated fibrinolysis. Those patients whose inhibitory capacity is adequate show no increased fibrinolytic activity, even if tpa antigen is markedly elevated. Patients with insufficient inhibitor exhibit increased fibrinolytic activity proportional to the amount of tpa activity that escapes inhibition. This interpretation is supported by the finding that tpa inhibitor activity was lowest in group 1 and highest in group 3 and correlated significantly with the DWBCLT.

Total tpa antigen in plasma is the sum of free tpa and tpa in complex with its inhibitors.¹⁹⁻²¹ In attempting to directly measure the tpa antigen level in whole plasma using the ELISA technique of Bergsdorf et al,¹⁵ however, we found that the apparent level of tpa antigen was not independent of the dilution of plasma assayed, with higher dilutions producing higher apparent tpa antigen levels (data not shown). In contrast, use of euglobulin precipitates resulted in tpa antigen levels that were independent of the dilution of euglobulin fraction assayed. In agreement with other investigators¹⁶ we also found that purified tpa added to plasma was quantitatively recovered in the euglobulin precipitate over a wide range of concentrations. Thus, it is likely that both free tpa and tpa bound to the inhibitor are detected with equal efficacy in the euglobulin fraction using the ELISA of Bergsdorf et al.¹⁵

There is as yet no agreement on the optimal method for measuring tpa inhibitor activity in plasma. Current assays are not standardized and may have methodologic limitations.²² The method we have employed, in which a single concentration of purified tpa is added to plasma, has been criticized on the grounds that the amount of tpa inhibitor activity in plasma increases as increasing amounts of tpa are added.²² Nonetheless, the finding that tpa inhibitor by this assay correlates strongly with both overall fibrinolytic activity and free tpa activity in patients with cirrhosis supports its usefulness in evaluating tpa inhibitory capacity of plasma. This type of tpa inhibitor assay has also proven useful in the

Table 2. Fibrinolytic Assays (Mean $\pm$ SD)

	Group 1	Group 2	Group 3	Normal
DWBCLT (minutes)	104 $\pm$ 35	176 $\pm$ 26	>240	>240
TPA antigen (ng/mL)	29.4 $\pm$ 25.9	30.9 $\pm$ 20.7	22.1 $\pm$ 13.9	5.2 $\pm$ 2.6
TPA activity (ng/mL)	7.4 $\pm$ 12.1	0.1 $\pm$ 0.2	0	0
TPA inhibitor (ng/mL)	16.2 $\pm$ 10.5	25.3 $\pm$ 15.8	32.0 $\pm$ 18.7	41.9 $\pm$ 9.8
a2PI (%)	53 $\pm$ 22	52 $\pm$ 25	77 $\pm$ 26	73-119%

study of impaired fibrinolysis in atherosclerotic heart disease.²³

Tpa inhibitors appear to be synthesized by endothelium, platelets, and placenta.²⁴⁻²⁶ Normal plasma may contain both endothelial and nonendothelial tpa inhibitors²⁷; the assay method of Juhan-Vague et al¹⁷ measures only the total tpa inhibitory activity in plasma and does not distinguish among the various inhibitors present. Thus, we cannot determine which type of tpa inhibitor is most important in the regulation of fibrinolytic activity in patients with liver cirrhosis. Moreover, since the factors controlling tpa inhibitor synthesis and release are not well defined, it is not yet possible to explain why some patients with cirrhosis are able to counteract increased levels of circulating tpa with increased levels of inhibitor, while others are not. Studies of the extent of protein C activation in cirrhosis may be relevant, since there is evidence that activated protein C inhibits tpa inhibitor release from cultured endothelium.²⁸ Thrombin may also be an important factor, since subclinical disseminated intravascular coagulation is common in liver cirrhosis,^{29,30} and thrombin has been shown to stimulate release of both tpa antigen and inhibitor by cultured endothelium.³¹

We found mean a2PI to be significantly reduced in patients with cirrhosis and accelerated fibrinolysis and normal in patients with cirrhosis and normal fibrinolysis, suggesting a significant contributing role for reduced a2PI in the pathogenesis of accelerated fibrinolysis in cirrhosis. Although a2PI did not correlate with the DWBCLT, the DWBCLT method we used may be insensitive to the inhibitory activity of a2PI, since it involves clotting whole blood in the absence of calcium, thus interfering with the cross-linking of a2PI to fibrin by factor XIII.³² In contrast to a previous study,⁸ we also found no correlation between a2PI and serum albumin, suggesting that factors other than hepatic synthetic rate are important in determining the level of a2PI in cirrhosis.

In addition to increased tpa activity, the possible contribution of increased urokinase-like plasminogen activator activity⁷ to the pathogenesis of accelerated fibrinolysis in cirrhosis needs to be considered. Since tpa inhibitors have also been reported to inhibit urokinase,^{9,17,24,26} reduced tpa inhibitory activity in cirrhosis may explain the apparently conflicting reports of increased urokinase-like activator activity⁷ and normal urokinase antigen³³ in patients with cirrhosis.

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