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Plasminogen activators in alcoholic cirrhosis: demonstration of increased tissue type and urokinase type activator

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SUMMARY Plasma samples from patients with alcoholic cirrhosis were analysed for plasminogen activators and for inhibitors of the fibrinolytic system. Plasminogen activator activity was considerably increased in patients' plasma compared with normal. Immunochemical characterisation of these plasminogen activators showed that they included both tissue type and urokinase type plasminogen activator. The major inhibitor of plasmin, α_2-antiplasmin, was decreased in the patients, but no evidence for the generation of plasmin was found.

It has been known for many years that abnormally enhanced fibrinolysis is responsible for some of the haemorrhagic problems associated with hepatic cirrhosis.[1,2] In 1964 Fletcher *et al*[3] showed that the in vivo clearance rate for plasma plasminogen activator after nicotinic acid injection was four times slower in cirrhotic patients than in normal controls. They suggested that abnormal fibrinolysis in cirrhosis may be due to failure of hepatic clearance mechanisms for plasminogen activator.

The primary plasma inhibitor of plasmin α_2-antiplasmin is decreased in liver disease.[4] Histidine rich glycoprotein, which is an inhibitor of fibrinolysis,[5] is similarly affected.[6,7] These observations provide additional or alternative explanations for the fibrinolytic activity seen in hepatic cirrhosis.

The technique of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by zymography on fibrin agarose layers,[8] has been applied to the analysis of plasma samples for plasminogen activators.[9] Using this approach, together with other methods, we have studied the fibrinolytic system in a group of patients with alcoholic cirrhosis. The aims of the study were: (*a*) to examine the nature of plasminogen activators responsible for increased fibrinolytic activity in cirrhosis; and (*b*) to investigate the possibility that plasmin generation occurs in the circulation and might, by consuming fibrinolytic inhibitors, be responsible for the reduced levels of these inhibitors seen in this disorder.

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Material and methods

Blood samples were collected into 0·1 volume of 0·13M sodium citrate and immediately cooled on ice. Platelet poor plasma was prepared by centrifugation at 1200*g* for 15 min at 4°C. Plasma samples were stored at −70°C.

Clottable fibrinogen was measured by a modification[10] of the method of Ratnoff and Menzie.[11] Plasminogen, α_2-antiplasmin, antithrombin III, histidine rich glycoprotein, α_1-antitrypsin, α_2-macroglobulin, and Cl inactivator were determined by electroimmunoassay.[12] Antiserum to histidine rich glycoprotein was kindly provided by Dr N Heimburger, Behringwerke AG. Crossed immunoelectrophoresis against antiserum to α_2-antiplasmin was performed as described previously.[13,14] In some studies purified human plasminogen[15] was incorporated into the agarose gel in the first dimension at a final concentration of 0·1 mg/ml to distinguish between plasminogen binding and non-binding forms of α_2-antiplasmin.[16]

The overall fibrinolytic activity of plasma was assessed by assay of unfractionated plasma on plasminogen replete fibrin plates.[14,17] The euglobulin clot lysis time was measured as described previously.[18]

Plasma plasminogen activators were fractionated on lysine-Sepharose, essentially as described by Radcliffe and Heinze.[19] Plasma samples (2ml) were loaded on to columns (0·8 × 3·5 cm), equilibrated with 50mM Tris, 1mM benzamidine, 1mM edetic acid pH7·5, and eluted with 1·5M NaCl followed by

0·2M e-aminocaproic acid, each dissolved in this buffer.

SDS-PAGE with zymography on fibrin agarose layers was by the method of Granelli-Piperno and Reich.[8] Samples (10μl) were prepared for SDS-PAGE by incubation with 10μl of 8M urea, 4% sodium dodecyl sulphate, 40mM iodoacetamide, 0·2M Tris pH8·0, for 30 min at 37°C and then made 10% with respect to glycerol. They were applied to a Laemmli[20] gel (separating and stacking gels were 10% and 3% acrylamide respectively; gel was 180 × 200 × 1·2mm) and run at 20mA for 3·5 h. The gel was washed for 1 h in 1 litre of 2·5% aqueous Triton X-100 with constant agitation and rinsed repeatedly with distilled water before applying to a 1·3mm thick fibrin agarose layer (Kabi fibrinogen, either containing plasminogen or depleted of plasminogen on lysine-Sepharose[15] 2mg/ml; thrombin 0·06U/nml, 17·5mM NaCl, 60mM Tris pH7·8, 0·8% agarose; final concentrations). After incubation overnight at 37°C the polyacrylamide gel was removed and the fibrin agarose layer, with activity apparent as bands of lysis, was photographed. When required, immunoglobulin to tissue type plasminogen activator or to urokinase (generously provided by Dr S Cederholm-Williams, John Radcliffe Hospital, Oxford) was incorporated into a section of the fibrin agarose gel.

PATIENTS

Eleven patients with alcoholic hepatic cirrhosis were studied. The diagnosis was established on the basis of a clear history, with physical signs and serum biochemistry typical of advanced hepatocellular damage and with liver biopsy features indicative of this diagnosis. All had haemorrhagic features of varying degrees of severity, ranging from cutaneous purpura to recent major gastrointestinal bleeding.

Results

Results of the laboratory investigations on the patients are summarised in the Table. All had evidence of active fibrinolysis, with lysis of fibrin plates by unfractionated plasma, in contrast to normal plasma. Euglobulin lysis time was variable between fast and normal. Plasma concentrations of α_2-antiplasmin and antithrombin III were low. Histidine rich glycoprotein concentration was low in most of the patients. Other plasma protease inhibitors, Cl inactivator, α_2-macroglobulin, and α_1-antitrypsin, were normal or raised. Plasma plasminogen concentrations were low, while values for fibrinogen were normal.

No complex of α_2-antiplasmin was found. This contrasts with other conditions with comparably low α_2-antiplasmin concentrations and similarly enhanced activity on fibrin plates, such as disseminated intravascular coagulation or primary hyperfibrinolysis (Fig. 1).[13 14 18] Crossed immunoelectrophoresis of α_2-antiplasmin with plasminogen incorporated into the gel was normal (Fig. 2), indicating a normal ratio of plasminogen binding and non-binding forms.

Plasma samples were analysed on SDS-PAGE, followed by zymography. Fig. 3 shows the pattern of plasminogen activator activities; the patients' plasma samples showed increased plasminogen activator activities compared with normal plasma. The major bands of activity in cirrhotic plasma had molecular weights of about 50 and 95K. These bands could be inhibited by antisera to tissue type plasminogen activator or to urokinase (Fig. 4); the 50K band was related to urokinase while all the other activities, including the 95K band, were related to tissue type plasminogen activator.

Plasma from normal subjects and from patients

Results of laboratory investigations on the patients

Patient	*Albumin (g/l)*	*British corrected ratio*	*Platelet count (× 10⁹/l)*	*Euglobulin lysis time (min)*	*Plasma fibrin plate lysis (mm diam)*	*α_2-antiplasmin (% normal)*	*Antithrombin III (% normal)*	*Histidine rich glycoprotein (% normal)*	*Fibrinogen (mg/100ml)*	*Plasminogen (% normal)*
1	23	2·3	100	140	10·5	33	14	25	383	23
2	23	1·6	112	140	11	38	26	25	290	38
3	25	3·1	90	220	8·5	63	55	79	336	48
4	30	1·3	122	240	5	44	52	125	204	43
5	27	1·5	52	180	8	50	42	21	260	28
6	29	1·5	50	75	10	46	46	67	255	65
7	35	1·5	162	55	19	46	46	61	315	74
8	28	2·5	134	55	17	42	34	83	245	38
9	23	2·2	279	300	8	42	53	82	200	50
10	33	1·1	177	>360	5	66	61	158	430	78
11	30	1·0	125	265	5	60	70	158	340	80
Normal controls	37–47	1·0	150–300	90–240	nil	100	100	100	200–350	100

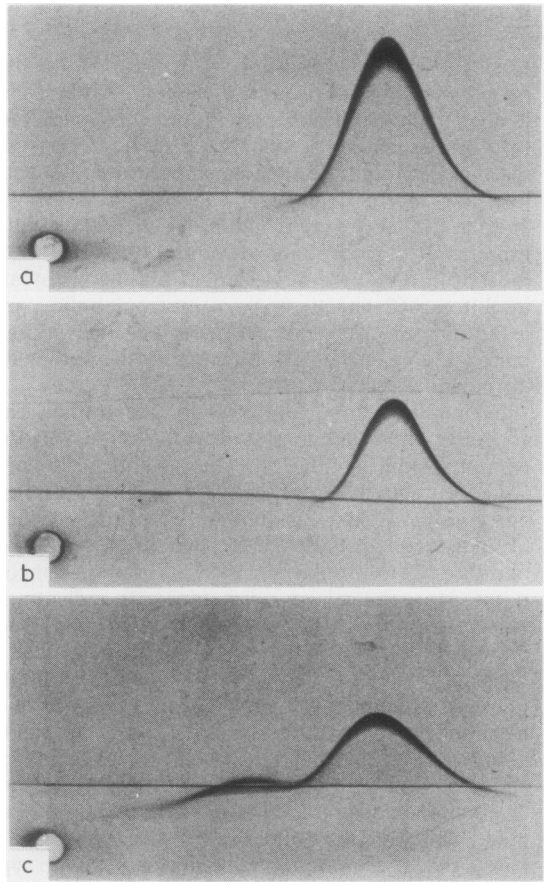

Fig. 1 *Crossed immunoelectrophoresis of α_2-antiplasmin. (a) Normal plasma; (b) plasma from patient 7; (c) plasma from a patient with primary fibrinolysis.*[18]

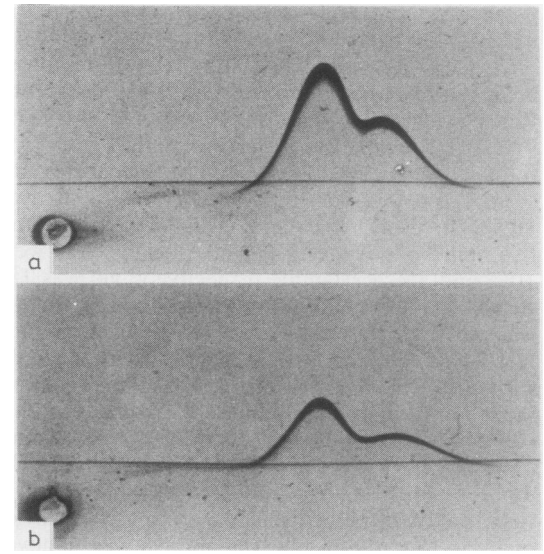

Fig. 2 *Crossed immunoelectrophoresis of α_2-antiplasmin with plasminogen in the first dimension. (a) Normal plasma; (b) plasma from patient 7.*

was fractionated on lysine-Sepharose and the fractions assayed for plasminogen activator activity on fibrin plates. Assay by this method showed a single peak of activity, eluted by 1·5M NaCl, when patients' plasma was fractionated (Fig. 5). No activity was seen by this method when normal plasma was fractionated. The fractions were also analysed by SDS-PAGE and zymography. The fractions from normal plasma contained 95K tissue type plasminogen activator in both the unbound peak and the 1·5M NaCl eluate (data not shown). The unbound fractions from patients' plasma contained both 50K urokinase related activity and tissue type plasminogen activator related activity of molecular weight 95K and lower (Fig. 5). The activity eluted with 1·5M NaCl consisted mainly of 95K and 65K material with a minor band of about 160K. All these bands were related to tissue type plasminogen

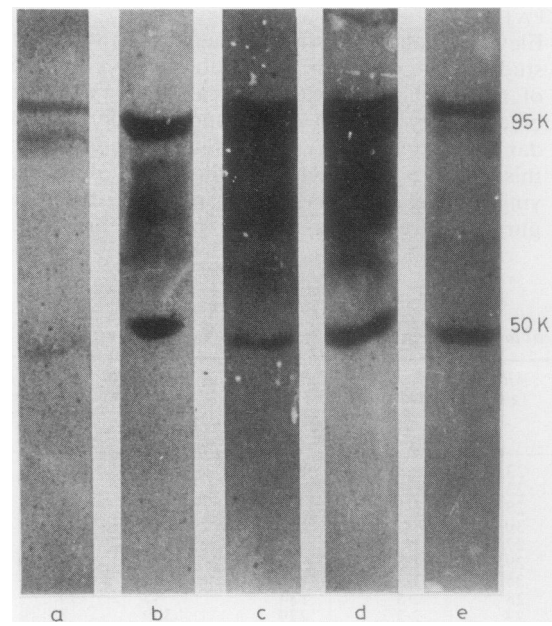

Fig. 3 *Plasma samples analysed by SDS-PAGE and zymography. (a) Normal plasma; (b, c, d, e) plasma from patients 1, 3, 5, and 6 respectively.*

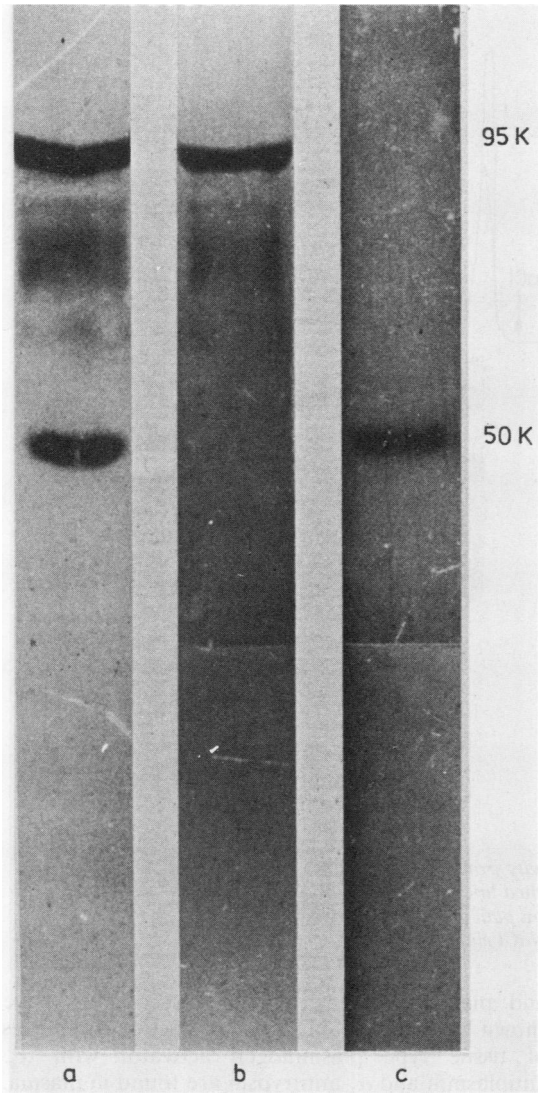

Fig. 4 *Plasma from 1 patient analysed by SDS-PAGE and zymography: inhibition by γ-globulins to urokinase type plasminogen activator and tissue type plasminogen activator incorporated in the fibrin agarose gel. (a) No γ-globulin; (b) γ-globulin to urokinase type plasminogen activator; (c) γ-globulin to tissue type plasminogen activator.*

activator. All the activity eluted from the lysine-Sepharose column was dependent on the presence of plasminogen; fibrin agarose layers prepared with plasminogen free fibrinogen showed no lysis.

Discussion

Plasma fibrinolytic activity is increased in patients with alcoholic cirrhosis. The mechanism underlying this increase remains open to debate. There is evidence that the liver has a role in the clearance of plasminogen activator from the circulation[3 21] and slow clearance of activator in cirrhotic patients is one explanation for the high fibrinolytic activity in their plasma. Alternatively, low concentrations of inhibitors of the fibrinolytic system, α_2-antiplasmin and histidine rich glycoprotein, have been suggested as a cause of the increase in fibrinolytic activity.[4 6 7 22]

In this study the question of increased plasma concentrations of plasminogen activator in liver disease was re-examined in the light of current knowledge of the various types of plasminogen activator which exist in blood. The high plasma concentrations of plasminogen activator, noted previously in hepatic cirrhosis,[3 23] were confirmed, and the study was extended to examine the nature of the activator(s) present. The earlier studies measured overall concentrations of plasminogen activator and did not discriminate between different types. Additionally, the methods used previously measured activator in fractions of plasma (prepared specifically to exclude proteins such as protease inhibitors from the assay systems) rather than in whole plasma; this may have excluded material from study. In this study the use of SDS-PAGE with zymography had the advantage of allowing examination of whole plasma. No material was excluded from the system and the different activators were separated from one another and from potential inhibitor proteins in the system itself, allowing demonstration of each plasminogen activator present. The activators in the plasma of the patients studied could be classified as tissue type plasminogen activator or urokinase type plasminogen activator on the basis of inhibition by specific antiserum to these activators. It is clear that both types of plasminogen activator are considerably raised in these patients. Another recent study has shown an increase in tissue type plasminogen activator,[24] using different techniques, but this is to our knowledge the first demonstration of increased urokinase type plasminogen activator in hepatic cirrhosis.

Separation of plasma plasminogen activators on lysine-Sepharose, followed by assay of the column fractions on fibrin plates, indicates that all the activity of patients' plasma was bound to lysine-Sepharose and could be eluted with 1·5M NaCl. This is in agreement with Radcliffe and Heinze,[19] who assayed fractions from post-exercise plasma on fibrin plates and found all the activity in the 1·5M NaCl wash. When the fractions from cirrhotic plasma were analysed by SDS-PAGE, followed by zymography, activity was apparent in the unbound fractions as well. The unbound activity included

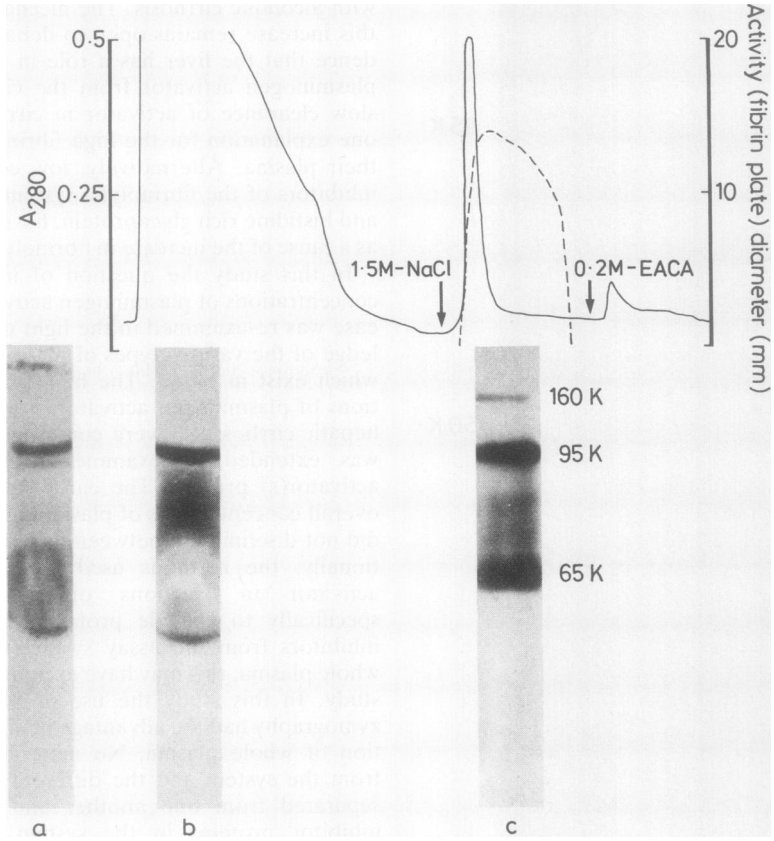

Fig. 5 *Elution of plasminogen activator activity from lysine-Sepharose: analysis of plasma from patient 7. Solid line: A_{280nm}; dashed line: activity on fibrin plates. SDS-PAGE and zymography. (a) plasma from patient 7; (b) peak fraction of unbound protein; (c) peak fraction of 1·5M NaCl eluate.*

urokinase type and tissue type plasminogen activators. These activities may be masked by inhibitors which are also unbound, so that no plasminogen activator activity is observed on fibrin plates. In the case of the tissue type plasminogen activator another interpretation is possible. Since this material has a molecular weight of about 95K, it may represent a complex which is inactive on fibrin plates but is reactivated under the conditions used for zymography, possibly by exposure to Triton X-100.[25]

The fractions eluted from lysine-Sepharose with 1·5M NaCl were active on fibrin plates. On analysis by SDS-PAGE and zymography the activity was all related to tissue type plasminogen activator. From the molecular weights of 65K, 95K, and 160K, it seems likely that these are due to free tissue type plasminogen activator (65K) and to two species of complex between tissue type plasminogen activator and plasma proteins. Rijken and others[26] have shown by other techniques that inactive complexes of tissue type plasminogen activator with α_2-antiplasmin and α_1-antitrypsin are found in plasma. The species of tissue type plasminogen activator which we have observed may be due to such complexes or to complexes with other inhibitors such as antiactivator.[27]

Early studies indicated that overall plasma inhibition of fibrinolysis was reduced in cirrhotic patients.[3,22] Subsequent studies have separately shown reduced levels of plasma α_2-antiplasmin,[4] antithrombin III,[28] and histidine rich glycoprotein.[6,7] The level of plasma plasminogen was also decreased.[3,22,23] In this study, we have seen this range of changes in the plasma proteins of cirrhotic patients and have confirmed that striking decreases in α_2-antiplasmin, antithrombin III, histidine rich glycoprotein and plasminogen occur. Our findings

indicate that in contrast to these reduced levels, the levels of the inhibitors α_2-macroglobulin, α_1-antitrypsin, and Cl inactivator are normal or raised.

Despite the high level of plasminogen activator and the reduction of α_2-antiplasmin concentration seen in this study, we found no evidence of plasmin generation in the circulation, as judged by crossed immunoelectrophoresis against antiserum to α_2-antiplasmin, which can detect the generation of complexes between plasmin and α_2-antiplasmin.[13 29] While this technique is relatively crude, it has been capable of demonstrating plasmin generation in other groups of patients with similarly reduced plasma concentrations of α_2-antiplasmin and comparable overall fibrinolytic activity.[14 18 30] This raises questions about the control of fibrinolysis in cirrhotic patients and indicates that while these patients may have a high potential for plasmin generation, owing to high levels of plasminogen activator and low α_2-antiplasmin concentrations, little such generation of plasmin actually occurs in the circulating blood, in contrast with the situation seen in other disorders.[14 18 30]

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