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, $\alpha_2$-antiplasmin, was decreased in the patients, but no evidence for the generation of plasmin was found.

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 1200g for 15 min at 4°C. Plasma samples were stored at $-70^\circ$C.

Clottable fibrinogen was measured by a modification of the method of Ratnoff and Menzie. Plasminogen, $\alpha_2$-antiplasmin, antithrombin III, histidine rich glycoprotein, $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, and Cl inactivator were determined by electroimmunodiffusion. Antiserum to histidine rich glycoprotein was kindly provided by Dr N Heimburger, Behringwerke AG. Crossed immunoelectrophoresis against antiserum to $\alpha_2$-antiplasmin was performed as described previously.

In some studies purified human plasminogen 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 $\alpha_2$-antiplasmin.

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

Plasma plasminogen activators were fractionated on lysine-Sepharose, essentially as described by Radcliffe and Heinze. Plasma samples (2ml) were loaded on to columns (0.8 x 3.5 cm), equilibrated with 50mM Tris, 1mM benzamidine, 1mM edetic acid pH 7.5, and eluted with 1-5M NaCl followed by

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0.2 M 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.* Samples (10 μl) were prepared for SDS-PAGE by incubation with 10 μl of 8 M urea, 4% sodium dodecyl sulphate, 40 M M iodoacetamide, 0.2 M Tris pH8.0, for 30 min at 37°C and then made 10% with respect to glycerol. They were applied to a Laemmli 2 gel (separating and stacking gels were 10% and 3% acrylamide respectively; gel was 180 × 200 × 1.2 mm) and run at 20 mA 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 1.3 mm thick fibrin agarose layer (Kabi fibrinogen, either containing plasminogen or depleted of plasminogen by lysine-Sepharose* 2 mg/ml; thrombin 0.06 U/ml, 17.5 mM NaCl, 60 mM 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 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 α2-antiplasmin and antithrombin III were low. Histidine rich glycoprotein concentration was low in most of the patients. Other plasma protease inhibitors, C1 inactivator, α2-macroglobulin, and α1-antitrypsin, were normal or raised. Plasma plasminogen concentrations were low, while values for fibrinogen were normal.

No complex of α2-antiplasmin was found. This contrasts with other conditions with comparably low α2-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 α2-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 antiserum 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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Albumin (g/l)</th>
<th>British corrected ratio</th>
<th>Platelet count (×1012/l)</th>
<th>Euglobulin lysis time (min)</th>
<th>Plasma fibrin plate lysis (mm diam)</th>
<th>α2-antiplasmin (% normal)</th>
<th>Antithrombin III (% normal)</th>
<th>Histidine rich glycoprotein (% normal)</th>
<th>Fibrinogen (mg/100 ml)</th>
<th>Plasminogen (% normal)</th>
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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 activator activity.

Fig. 1 Crossed immunoelectrophoresis of α2-antiplasmin. (a) Normal plasma; (b) plasma from patient 7; (c) plasma from a patient with primary fibrinolysis.18

Fig. 2 Crossed immunoelectrophoresis of α2-antiplasmin with plasminogen in the first dimension. (a) Normal plasma; (b) plasma from patient 7.

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.
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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 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, α2-antiplasmin and histidine rich glycoprotein, have been suggested as a cause of the increase in fibrinolytic activity.

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, 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 antisera 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, 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 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. 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...
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.

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 have shown by other techniques that inactive complexes of tissue type plasminogen activator with α2-antiplasmin and α1-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.

Early studies indicated that overall plasma inhibition of fibrinolysis was reduced in cirrhotic patients. Subsequent studies have separately shown reduced levels of plasma α2-antiplasmin, antithrombin III, and histidine rich glycoprotein. The level of plasma plasminogen was also decreased. In this study, we have seen this range of changes in the plasma proteins of cirrhotic patients and have confirmed that striking decreases in α2-antiplasmin, antithrombin III, histidine rich glycoprotein and plasminogen occur. Our findings...
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References


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