Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 162-168

IDENTIFICATION OF TWO TYPES OF PROTEIN IMMUNOCHEMICALLY RELATED TO URINARY UROKINASE OCCURRING IN HUMAN PLASMA

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Received August 22, 1986

SUMMARY. Plasma urokinase, a plasminogen activator immunochemically related to urinary urokinase (UK), was removed from human plasma (3.5 ng/ml) by immunodepletion with antibodies raised against UK. The remaining plasminogen activator activity of the depleted plasma could not be inhibited by anti-UK antibodies and a sensitive ELISA for UK did not detect any UK levels that were higher than the background of the assay (0.1 ng/ml). However, when the depleted plasma was subjected to SDS-PAGE, substantial amounts of protein were found hereafter around 110 and 46 kD which now gave a positive reaction in the ELISA (35-350 ng/ml plasma). From these observations it is concluded that in human plasma two types of UK-related protein occur: Type I, among which the plasma urokinase, has antigenic determinants which are directly accessible to the anti-UK antibodies, Type II has determinants in a latent form. The function of the 110 kD type-II protein is that of a plasminogen activator; that of the 46 kD protein is not yet clear.

INTRODUCTION. Urokinase is a plasminogen activator originally recovered from urine and later from culture media of kidney cells (1,2). More recently the suggestion of Shakespeare and Wolf (3) that urokinase is present in blood was confirmed (4,5,6). It appeared to be identical (7) to the previously described Factor XII-independent plasminogen activator (8). Apart from the urokinase-type activator, two other plasminogen activators occur in plasma (9): 1. the tissue--type plasminogen activator (t-PA) as described by Rijken et al. (10) and by Collen's group (11) and 2. the postulated (12), but as yet not identified, factor XII-dependent plasminogen activator. During our attempts to identify and

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Abbreviations: UK, urokinase; t-PA, tissue-type plasminogen activator; HWM-UK, high molecular weight urokinase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; DEF, dextran sulphate euglobulin fraction; AUK, anti-urokinase; BAU, blood activator units; IgG, immunoglobulins type G.

0006-291X/87 $1.50

characterize the latter activator, we observed that plasma, immunodepleted in UK and t-PA, still contains substantial amounts of UK related antigen, but in a latent form only detectable after SDS treatment and separation of polypeptides on PAGE. These findings are described below.

MATERIALS AND METHODS. The following materials were obtained from the indicated sources:
Flufenamic acid from Aldrich Europe, Beerse, Belgium, low molecular weight markers for electrophoresis from Pharmacia Ltd., Uppsala, Sweden and urinary Urokinase (a mixture of MW 55,000 and 33,000 UK) from Leo Pharmaceutical Industries, Ballerup, Denmark.
All other chemicals used were of analytical grade from Merck, Darmstadt, West Germany.
Rabbit anti-UK IgG's were isolated from antisera raised against HMW-UK as described by Wijngaards et al. (5).
Rabbit anti-t-PA IgG's were a gift of Dr. J.H. Verheijen from our Institute.
Platelet-poor citrated pooled plasma was prepared as described by Kluft et al. (13).
UK-depleted plasma was prepared using AUK antibodies coupled to Sepharose-4B as described by Kluft et al. (7).
Plasminogen activator activity assay: Plasminogen activator activity of urinary UK and of plasma was assayed with the standardized fibrin plate method (14). Activity of plasma was determined in DEF's in the presence of 2 mM sodium flufenamate to activate proactivators and to prevent the possible effects of inhibitors as described by Kluft et al. (15). Plasminogen activator activity was measured as the area of the lysed zones obtained in 17 hrs. In the case of urinary UK the results are expressed in IU/ml by comparison with the WHO International Standard for UK (code 66/46). In the case of DEF's the area's were, after conversion to BAU/ml as described by Kluft et al. (15), expressed in IU/ml (100 BAU/ml corresponding to 0.7 IU of UK activity per ml (16)).
SDS-PAGE and extraction of gel slices: SDS-PAGE was carried out on 14.5 x 12 x 0.1 cm gel slabs according to Laemmli (17) with a 5% stacking gel and 8.5% running gel. The unreduced samples, mixed with an equal volume of sample buffer, were applied to the slab gel and electrophoresed overnight at 3 mA. After completion of the electrophoresis, lanes were cut out and washed for 2 hours at 37°C in 2.5% Triton X-100. Then the lanes were sliced (27 slices of 0.32 cm) and the slices were extracted by incubating for 48 hours with 200 µl 0.01 M sodium phosphate buffer pH 7.4 containing 0.14 M NaCl, 5 mM EDTA, 1 mg/ml bovine albumin, 0.01% Tween 80 and 0.02% NaN_3.

For protein staining gel lanes were stained with 2% Coomassie Brilliant Blue R250 in 30% methanol, 10% acetic acid and destained in 30% methanol, 10% acetic acid. Molecular weights were calculated using low molecular weight markers electrophoresed in parallel lanes.
Assay of UK and t-PA antigen: UK antigen in the gel slice extracts was determined with the ELISA described by Binnema et al. (18), t-PA antigen was determined with the enzyme immunoassay described by Rijken et al. (19).

RESULTS AND DISCUSSION. Fig. 1 (curve 1) shows that 50% of the plasminogen activator activity of human plasma at rest can be inhibited by anti-UK IgG. Under the same conditions the activity of urinary urokinase (curve 2) is fully inhibited. The remaining 50% of activity is still present in the immunodepleted

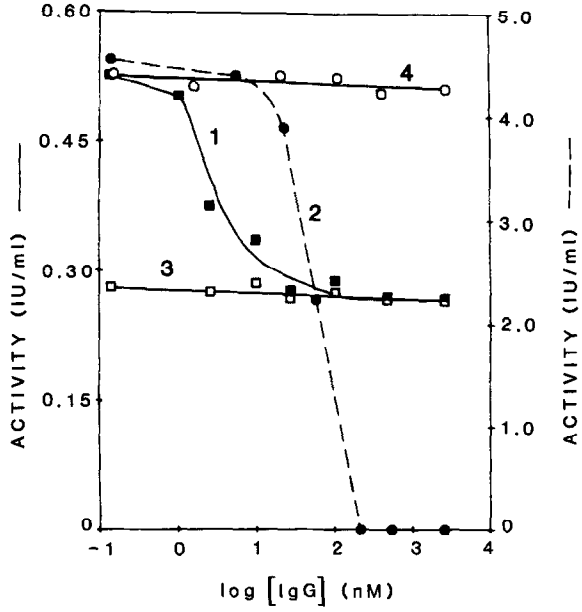

Figure 1. Inhibition of plasminogen activator activity by increasing amounts of AUK and anti-t-PA antibodies. Plasminogen activator activity was measured by the fibrin plate method (as described in Materials and Methods). Five microliters of different dilutions of an AUK IgG stock (concentration 16.5 µM, assuming $E^{1\%}_{280}$ = 15.0) were added to 25 µl of a concentrated DEF (120%) of pooled normal plasma (curve 1) or UK depleted plasma (curve 3) and to 25 µl urinary UK (100 m IU) (curve 2). In curve 4, 5 µl of different dilutions of an anti-t-PA IgG stock (concentration 15,5 µM) were added to 25 µl of a concentrated DEF (120%) of pooled normal plasma. Then the drops were applied to the fibrin plate. On the ordinate the plasminogen activator activity is expressed in IU/ml. The concentration of IgG's on the abscissa is that in the drops before application to the fibrin plates.

plasma (curve 3). This implies that 1. the plasminogen activator activity of the species removed by the immunodepletion (plasma urokinase) was 100% inhibited by the anti-UK antibodies and 2. the remaining activity is due to another plasminogen activator. In agreement, an ELISA for UK (18) detected 3.5 ng/ml plasma before the immunodepletion and did not detect any UK levels higher than the background of the assay (0.1 ng/ml) after the depletion confirming 100% removal of plasma urokinase. Addition of antibodies against t-PA (curve 4) and immunodepletion of the plasma with anti-t-PA IgG's (not shown) had no effect, indicating that in the experiments of Fig. 1 t-PA was not involved.

The proteins remaining in the immunodepleted plasma were subjected to SDS-PAGE. Fig. 2A shows the protein staining. In Fig. 2B the extracts of the gel slices of a similar gel were searched for the presence of UK with the ELISA, although before the SDS-PAGE no UK could be detected. Quite unexpectedly

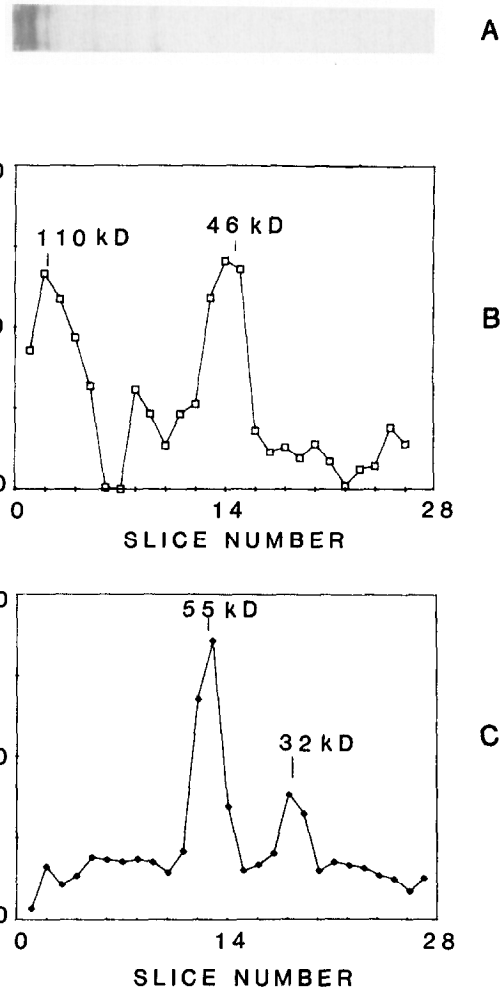

Figure 2. SDS-PAGE of urinary UK and of plasma immunodepleted in UK. Fifty microliters of a DEF of pooled plasma immunodepleted in UK (A,B) and 3 ng urinary UK (C) were subjected to SDS-PAGE (see Materials and Methods). Hereafter the protein was stained in (A), and in (B,C) the gels were sliced and extracted with buffer (see Materials and Methods). Subsequently, UK antigen (pg) was determined in the extracts (200 µl) of the gel slices with the ELISA.

substantial amounts of UK-related protein were found around 110 and 46 kD. The integrated areas under the peaks account for 35 ng UK/ml plasma, which is 10 times more than the concentration of the previously removed plasma urokinase. In order to make corrections for possible losses during extraction of the gels a commercial UK preparation (mixture of MW 55,000 and 33,000 UK) was run and assayed under the same conditions (Fig. 2C). The integrated areas under the peaks accounted for only 10% of the UK applied to the gels. If the extraction in Fig. 2B occurred with the same low efficiency this implies that the estimat-

ed amounts of UK-related protein in the immunodepleted plasma even may increase up to 350 ng/ml. Possible nonspecificity of the ELISA under these conditions is unlikely since the prominent protein bands in Fig. 2A do not occur at 110 and 46 kD and the ELISA did not give any response if the second antibody, the goat anti-UK, was omitted. Assay of the extracts of the gel slices with an ELISA for t-PA showed that the proteins at 110 and 46 kD are not related to t-PA. Possible artifacts caused by the immunodepletion procedure are excluded, since the 110 and 46 kD proteins also could be detected after SDS-PAGE of undepleted plasma.

The above results indicate that two types of UK-related proteins occur in human plasma. First the type of protein with UK-related antigenic determinants directly accessible to the anti-UK antibodies (plasma-urokinase). We will denote this type of protein by type-I urokinase.

Secondly the type of protein which, in the untreated plasma, has UK-related antigenic determinants present in a latent form. These antigenic determinants become accessible to the anti-UK antibodies only after SDS treatment and separation of the polypeptides on PAGE. We will denote this type by type-II urokinase.

The function of the type-I protein is obvious, being the factor XII-independent plasminogen activator, the plasma-urokinase, as identified earlier (8). Also the function of the 110 kD type-II protein is clear, since we demonstrated previously (20,21) that this protein indeed has plasminogen activator activity and is effective in fibrinolysis. However, by that time the complication of the two types of protein was not noticed and, because of its relatedness with UK, the 110 kD protein was thought to be the factor XII-independent plasminogen activator. Nowadays (v.i.) we know that this is not so and most recently we did experiments to be published elsewhere, which demonstrate that the 110 kD protein indeed is dependent on the presence of factor XIIa and kallikrein for its activation to a plasminogen activator and therefore may be identical with the postulated (12) factor XII-dependent plasminogen activator. Only the function of the 46 kD type-II protein has to be clarified yet.

Also the relation between the type-I plasminogen activator and the type-II proteins is most intriguing. At present we are investigating the following possibilities: 1. The type-II protein is a precursor form which can be chemically converted to the type-I protein. Such a relationship for instance has been demonstrated (22) for coagulation factors α-Factor XIIa (MW 80,000) and β--Factor XIIa (MW 28,000). 2. The type-II protein is the product resulting from chemical conversion of type-I protein, e.g. by irreversible reaction with inhibitors present in the plasma. The formation in plasma of irreversible complexes around 100 kD between plasminogen activator and plasma-inhibitor has been reported by several groups (23,24,25), but in those cases the plasminogen activator activity of the formed complex was an artifact resulting from the SDS-PAGE treatment (26,27,23) and not an activity, already present in the plasma before SDS-PAGE. 3. Type-I and -II proteins cannot be interconverted chemically, are the transcription products of different genes, but still contain homologous units (e.g. the kringle domains) as is the case in many of the proteases of blood coagulation and fibrinolysis (28). After the denaturing conditions of the SDS-PAGE the type-II proteins may now be unfolded in such a way that segments of homologous units give raise to some reactivity with the antibodies against type-I protein. The homology should be very strong, since no such a reactivity occurred with antibodies against t-PA, the thusfar closest related molecule to UK (28).

ACKNOWLEDGEMENTS. These investigations were supported in part by the Foundation for Medical Research FUNGO (grant nr. 900-526-058). Thanks are due to Drs. C. Kluft and F. Haverkate for critical reading of the manuscript and to Mrs. C and M. Horsting for typing the manuscript.

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