THE INTRINSIC SYSTEM OF FIBRINO

COMPONENTS AND MECHANISM

DOEDE J. BINNEMA

THE INTRINSIC SYSTEM OF FIBRINOLYSIS: COMPONENTS AND MECHANISM

STELLINGEN

1. Dat Koch en Antranikian korrelafbraak gemeten hebben bij incubatie van natief aardappelzetmeel met thermostabiele amylases bij een temperatuur van 70 °C lijkt onwaarschijnlijk.
   Koch en Antranikian, Starch 1990; 42: 397-403.

2. Het door Kinzy en Merrick beschreven tryptisch fragment van elongatiefactor 1α is onvoldoende gekarakteriseerd.
   Kinzy en Merrick, J Biol Chem 1991; 266: 4099-4105.

3. Het grote verschil in metastase vorming in naakte muizen na subcutane of intraveneuze inoculatie van tumorcellen, toont aan, dat het gebruik van slechts één van beide inoculatiemethoden beperkte waarde heeft voor de studie van metastasering.
   Axelrod c.s., Mol Cell Biol 1989; 9: 2133-2141.
   Quax, Proefschrift, Leiden 1991.

4. Bij de conclusie van Beckmann c.s. dat t-PA via het finger-domein aan FCB2-fragmenten van fibrine gebonden wordt, is er niet voldoende onderscheid gemaakt tussen resultaten behaald met fibrine en die met FCB2-fragmenten.
   Beckmann c.s., J Biol Chem 1991; 266: 2227-2232.

5. Bij het vaststellen van de hoeveelheid bacteriële celwandfragmenten met behulp van de muraminezuurbepaling volgens Hadzija, hebben Severijnen c.s ten onrechte geen rekening gehouden met het eveneens in deze preparaten aanwezige rhamnose.
   Severijnen c.s., J Rheumatol 1989; 16: 1601-1608.

6. De conclusie van Wersch c.s., dat er bij diabetes-patiënten een significante relatie bestaat tussen HbA_1 en D-dimeer, lijkt, gezien de getoonde gegevens, onwaarschijnlijk.
   Wersch c.s., Haemostasis 1990; 20: 241-250.

7. Het vervangen van voor ieder begrijpelijke bedieningsknoppen op eenvoudige laboratoriumapparatuur door microprocessor-gestuurde menu's met een veelvoud aan keuze-mogelijkheden, leidt niet tot een verhoging van het bedieningsgemak.

8. Het aanbrengen van geleiderichels tussen voor fietsers en voor automobilisten bestemde weggedeelten, verhoogt niet altijd de verkeersveiligheid. Waar deze voorzieningen aangebracht zijn en aanleiding geven tot ongelukken, dienen de aanbrengers verantwoordelijk gesteld te worden.

Stellingen bij het proefschrift: "The intrinsic system of fibrinolysis: components and mechanism".

Doede J. Binnema, juni 1991.

THE INTRINSIC SYSTEM OF FIBRINOLYSIS: COMPONENTS AND MECHANISM

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aan mijn ouders
voor Seerina en Annelies

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

In the human body the state of fluidity of the blood is maintained by a delicate balance between the systems of coagulation and fibrinolysis. After injury or perturbation of the vessel wall a haemostatic plug (fibrin clot) is needed to prevent blood loss. Deposition of fibrin is then regulated by the system of coagulation. The fibrin network, enforced by platelets, serves a temporary function and is in due time, after tissue repair, degraded by the fibrinolytic system into soluble fibrin degradation products (FDP) (1-3).

Both the coagulation and the fibrinolytic system are made up of a cascade of consecutive activation steps as depicted in Fig. 1 for the system of fibrinolysis. The lysis of a fibrin clot is catalyzed by the serine protease plasmin (Pl) which is generated from the zymogen plasminogen (Plg) by the action of plasminogen activators (PAs). Upon certain stimuli PA activity may be generated, either by secretion of active PAs from cellular sources into the blood or by proteolytic cleavage of PA-zymogens already present in the circulation (4, 5). The actual activity brought about by such a cascade is not only decided by the rate of generation of active components but also by the rate of interactions with inhibitors (Inh) present in blood. In healthy humans both the coagulation and fibrinolytic cascades will be in a steady state with low levels of active components. Impairment of the system of coagulation or excessive activation of the fibrinolytic system may lead to a bleeding tendency, whereas excessive coagulation or impaired fibrinolysis may lead to a thrombotic condition.

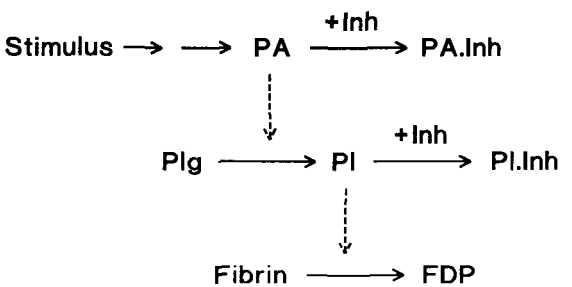

Figure 1. The fibrinolytic system: a cascade process.
Abbreviations: PA, plasminogen activator; Inh, inhibitor; Plg, plasminogen; Pl, plasmin; FDP, fibrin degradation products.
Symbols: solid arrows indicate chemical conversion; broken arrows, action of proteolytically active enzyme.

Research into the fibrinolytic system is one of the main topics of the Gaubius Laboratory IVVO TNO. Much attention is given to plasminogen activators, since changes in their availability and activity play a central role in the regulation of the fibrinolytic system in blood, and other body fluids (1, 3, 6, 7). Two immunologically distinct types of PA can be distinguished, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

t-PA is a glycoprotein with a M_r of about 70,000 that was originally discovered in vascular tissue (8) and later purified and characterized from uterine tissue (7, 9). It occurs as an active single- or two-chain molecule with a high affinity for fibrin (10-12). t-PA is a poor plasminogen activator by itself, but in the presence of fibrin its activity is enhanced about 100-fold (13). In this way plasminogen activation is restricted to the fibrin clot.

u-PA is also a glycoprotein. It was originally recovered from urine and subsequently also from culture media of kidney cells (14-16). The main molecular forms of u-PA found originally were two-chain molecules (tcu-PA) (17, 18) with molecular weights of about 55,000 and 33,000. Early studies also reported the presence of a zymogen form of u-PA which was later identified as a single-chain, M_r 55,000 molecule (scu-PA) (15, 19-23). Upon proteolytic cleavage of scu-PA by proteases such as trypsin or plasmin the active centre is disclosed (20, 22-25). Contrary to t-PA, tcu-PA is able to directly activate plasminogen in the circulation (26). In this case the fibrin selectivity is obtained by the focal activation process: scu-PA and fibrin-bound plasminogen are involved in a two-step cascade process with reciprocal feed-back activation, amongst others initiated by the low activity of scu-PA with respect to fibrin-bound plasminogen (27-31).

scu-PA constitutes part of the intrinsic system of fibrinolysis (Fig. 2) which involves Pas present in the circulation in the zymogen form (5, 6, 32-36). The potential activity of this system can be determined in vitro by activation of plasma with dextran sulphate under simultaneous removal of inhibitors by iso-electric precipitation (5, 6). In plasma taken from healthy man at rest scu-PA activity comprises about 50 blood activator units (BAU)/ml of the total potential activity of 100 BAU/ml (36), a further 15 BAU/ml comes from the PA activity of the activated contact factors prekallikrein (pKK) and factor XII (FXII) and the remainder is attributed to a recently identified (see Chapter VII of this thesis), contact-activation dependent, plasminogen proactivator (2, 5, 37, 38). t-PA activity is almost negligible,

due to the excess of free plasminogen activator inhibitor 1 (PAI-1) in blood (39).

After stimuli such as DDAVP infusion or exercise the plasma level of scu-PA is enhanced about two-fold and that of t-PA antigen increases about three-fold (40-43). By contrast the activity of t-PA is enhanced more than twenty-fold, since its plasma level is then well above that of PAI-1. This results in a net-rise of t-PA-activity in the in vitro measurement of about 100 BAU/ml (5, 44). This considerable enhancement of t-PA activity after stimulus, caused by release from the endothelial cells, originally led investigators to designate t-PA as "the extrinsic activator". In vivo the mechanism of clot lysis by the intrinsic and extrinsic activators is complementary and the effect of the combination may be synergistic (45-50). The concurrent enhancement of t-PA and scu-PA under stimulatory conditions may thus be of crucial importance.

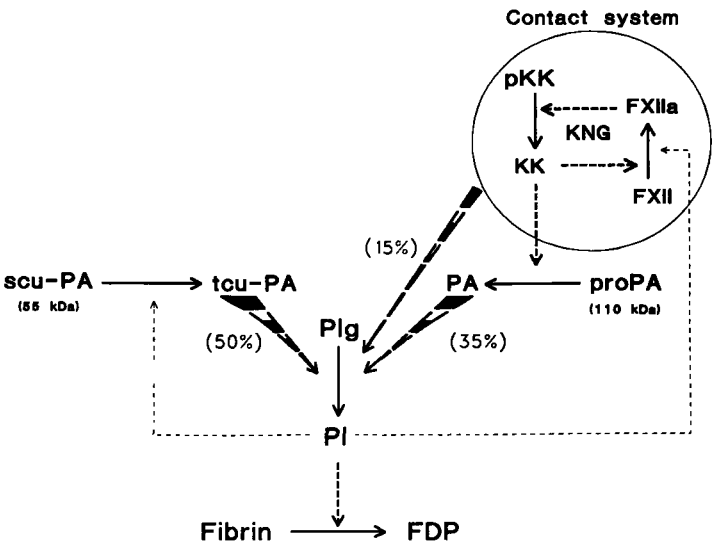

Figure 2. The intrinsic system of fibrinolysis.
Abbreviations: pKK, prekallikrein: KK, kallikrein; FXII, Factor XII; FXIIa, active Factor XII; KNG, kininogen; proPA, plasminogen proactivator; PA, plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; tcu-PA, two-chain urokinase-type plasminogen activator; Plg, plasminogen; Pl, plasmin; FDP, fibrin degradation products.
Symbols: solid arrows indicate chemical conversion; broken arrows, action of proteolytic active enzyme; broken lines, feed-back action of plasmin.

Because of its fibrin affinity and enhancement of activity after stimuli, in the past two decades much attention has been given to the extrinsic activator t-PA. The importance of the intrinsic system, which constitutes the predominant portion of potential activity in man at rest and may exceed the extrinsic portion even after exercise, has been recognized only recently.

In this thesis, the intrinsic system of fibrinolysis (see Fig. 2) was investigated in order to obtain a better understanding of this pathway of plasminogen activation. In CHAPTERS II and III the development is described of sensitive immuno-assays which may distinguish between the zymogen scu-PA, the active enzyme tcu-PA and the inhibitor-bound forms of u-PA. CHAPTER IV deals with the molecular forms and fibrin affinity of scu-PA and tcu-PA in plasma and in CHAPTER V an analysis was made of the activators of scu-PA in plasma. CHAPTER VI deals with the discovery in plasma of a second type of protein immunologically related to u-PA but with u-PA-related antigenic determinants in a cryptic form. In CHAPTER VII this second type of u-PA was identified as the contact-system dependent plasminogen proactivator (see Fig. 2). Based on the results presented in CHAPTERS II to VII, in CHAPTER VIII the opinion is offered that the different components of the intrinsic system may work together in a cascade of consecutive activation steps.

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CHAPTER II

QUANTITATION OF UROKINASE ANTIGEN IN PLASMA AND CULTURE MEDIA BY USE OF AN ELISA

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QUANTITATION OF UROKINASE ANTIGEN IN PLASMA AND CULTURE MEDIA BY USE OF AN ELISA

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ABSTRACT

An ELISA was set up using polyvinylchloride microtiter plates coated with rabbit anti-UK IgG's and affino-purified goat anti-UK IgG's as second antibody. Detection occurred with rabbit anti-goat IgG antibodies conjugated with alkaline phosphatase. The assay is specific for urokinase (UK) with a detection limit of 100 pg/ml sample. Tissue-type plasminogen activator, up to concentrations of 100 ng/ml, does not interfere. The assay measures the antigen of the inactive zymogen pro-UK, the active enzyme UK and the UK-inhibitor complex with equal efficiency and gives the total UK antigen present, irrespective of its molecular form. Culture media of fibroblasts, endothelial- and kidney cells showed, despite the absence of active UK, antigen levels of 1.2, 23 and 65 ng/ml, respectively. In human plasma the UK concentration was found to be 3.5 ± 1.4 ng/ml (*mean ± SD, n = 54*). The inter- and intra-assay variations were 20% and 6%, respectively.

INTRODUCTION

UK is a plasminogen activator originally recovered from urine and later from culture media of kidney cells (1,2). Recently, it was demonstrated that UK also occurs in blood (3,4,5), probably as an inactive zymogen and in complex with inhibitors (6). The fibrinolytic potential of UK in blood is high; after activation in vitro the active UK formed accounts for about 50% of the total fibrinolytic activity present in the blood (7). The question, whether this plasma UK originates from the kidney cells or from other sources has not been answered yet. Endothelial cells are also in contact with the blood-stream and are known to produce UK in culture (8,9). Until recently, UK concentrations in plasma and culture media were estimated by measurement of UK activity (4,10). However, incomplete activation of pro-UK and formation of inactive UK-inhibitor complexes could thereby result in an under-

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Key words: ELISA, urokinase, plasminogen activator, fibrinolysis, human plasma, culture media.

estimation of the UK concentration. This problem may be avoided using immunological techniques such as radioimmunoassays (5,11-13) and EIA's (14). The ELISA described in this report uses polyclonal affino-purified anti-UK antibodies, detects UK levels with a sensitivity of 3×10^{-18} mol and determines the different molecular forms of UK with an equal efficiency. This ELISA is applicable for the estimation of UK levels present in plasma and culture media. Part of this work has been presented before (15).

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: HMW-UK from Choay, Paris, France and from Winthrop Laboratories, New York, U.S.A. (Winkinase). Protein A-Sepharose, CH-Sepharose 4B and Sepharose 4B CNBr-activated from Pharmacia Fine Chemicals, Uppsala, Sweden. Rabbit anti-(goat IgG) IgG's alkaline phosphatase conjugate, p-nitrophenylphosphate, Tween-80 and bovine serum albumin (fraction V) from Sigma Chemical Company, St. Louis, U.S.A.; Titertek immunoassay plates from Flow Laboratories, Irvine, Scotland and aprotinin (trasylol) from Bayer, Leverkusen, F.R.G. All other reagents used were of analytical grade.

Antibodies against UK: Rabbit anti-UK IgG's and goat anti-UK IgG's were isolated from antisera raised against Choay UK as described by Wijngaards et al. (4).

Affinopurification of goat anti-UK IgG's: A Sepharose 4B column with immobilized HMW-UK was prepared by coupling Winkinase (purified according to Åstedt et al. (16)) to CNBr-activated Sepharose 4B according to the manufacturers' instructions. To this column goat anti-UK IgG's were absorbed and affinospecific antibodies were eluted with 0.1 M glycine/HCl, pH 2.8. After dialysis against 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4 (PBS) these antibodies had a concentration of 250 µg IgG's per ml (assuming $E^{1\%}_{280}$ = 15.0).

Tissue-type plasminogen activator (t-PA) obtained from a human melanoma cell culture (17) was a gift of Dr. J.H. Verheijen.

Plasminogen and plasmin were kindly provided by Dr. D.W. Traas from our Institute.

Pro-UK (MW 55,000) was purified from a monkey kidney cell culture as described by Wijngaards et al. (18).

Diisopropylfluorophosphate (DFP)-inactivated UK was prepared by twice incubating 2 ml of a solution of 10 IU/ml Choay UK with 20 ml 0.1 M DFP for two hrs at room temperature. After this treatment no urokinase activity could be detected by measurement of activity on the chromogenic substrate for UK pyro-Glu-Gly-Arg-pNA as described below.

Platelet-poor plasma was prepared at 4°C from citrated blood taken at rest and stored at -70°C as described by Kluft et al. (19).

Cell culture media from endothelial cells, Hep G2 cells, hepatocytes, Bowes melanoma cells and fibroblasts were kindly provided by Drs. Van Hinsbergh, Kooistra and Emeis of our Institute. Samples of media from a monkey kidney cell culture were kindly provided by Dr. A.L. Van Wezel, RIVM, Bilthoven, The Netherlands.

UK depleted media and -plasma were obtained by immunoadsorption with immobilized anti-UK IgG's as described by Kluft et al. (7).

Measurement of UK activity. Activity was determined using the chromogenic substrate for UK pyro-Glu-Gly-Arg-pNA (S-2444, Kabi, Stockholm, Sweden). 100 µl samples were incubated with 150 µl 0.1 M Tris/HCl, pH 8.8, containing 100 mM NaCl, 0.025% Tween-80, 40 KIU/ml aprotinin and 0.33 mM S-2444. All incubations were performed at 37°C in microtiter plates. The activity was measured with a Titertek Multiscan spectrophotometer (Flow Laboratories,

Irvine, Scotland) and expressed as the change in absorbance per hour at 405 nm. UK activity expressed in IU was obtained by comparison with the WHO International Standard for UK (code 66/46).

Assay of pro-UK. Pro-UK was determined by incubating samples with 2.5 µg/ml plasmin for 1 h at 37°C, then followed by measurement of UK activity as described above.

ELISA of urokinase. Polyvinylchloride microtiter plates containing 96 flat-bottomed wells were coated by overnight incubation with 200 µl 0.05 M sodium carbonate buffer, pH 9.6, containing 10 µg/ml rabbit anti-UK IgG's. The plates were stored at -20°C or used directly. Prior to use, the wells were washed three times with 200 µl PBS containing 0.01% Tween-80 and 0.02% NaN_3 (PBS-Tween) (20). All incubations were performed on a tilting table at room temperature while the plates were covered with parafilm. Incubation periods of 2 hrs appeared to be sufficient for optimal binding. Routinely, 150 µl of sample dilutions in PBS-Tween containing 5 mM EDTA and 1 mg/ml bovine albumin (Buffer A) were added to the coated wells and incubated for a six-hour period. Subsequently the wells were washed five times with 200 µl PBS-Tween and incubated overnight with 150 µl PBS-Tween containing 1 mg/ml bovine albumin and 0.8 µg/ml affinopurified goat anti-UK IgG's. After repeating the washing procedure, the wells were incubated for 2 hrs with 150 µl rabbit-anti(goat IgG) IgG's, conjugated with alkaline phosphatase, properly diluted in PBS-Tween containing 1 mg/ml bovine albumin. Again the wells were washed and then incubated with 150 µl p-nitrophenylphosphate (1 mg/ml) dissolved in 10% diethanolamine buffer, pH 9.8, containing 0.1 mg/ml $MgCl_2.6H_2O$ and 0.02% NaN_3. The absorbance at 405 nm was recorded during 2 hrs on a Titertek Multiscan spectrophotometer and the ΔOD/hr was extrapolated from the linear part of the curve. The amount of antigen in the various samples was calculated by reading the ΔOD/hr from calibration curves obtained with Choay UK. The calibration curve was linear for UK-concentrations up to 2 ng/ml (ng UK were calculated from the activity using a specific activity of 104.000 IU/mg (see ref. 21)). When non-immune rabbit or goat IgG's were used no dose-dependent binding of the UK antigen could be detected.

RESULTS AND DISCUSSION

VALIDATION

Background and detection limit. The background measured in immunodepleted plasma and -culture media, amounted 0.1 ΔOD/h, the same value as found in buffer. The smallest response statistically significantly different from this background (see Rodbard, ref. 22), amounts 0.2 ΔOD/h, corresponding to a detection limit of the assay of 100 pg UK/ml (150 µl sample), or 3×10^{-16} mol UK.

Specificity. In human plasma and culture media serine proteases, structurally related to UK, may occur. Especially t-PA, which also is a plasminogen activator, and the substrate plasminogen, which occurs in plasma in concentrations 10^4 times higher than UK, could interfere. Despite the structural resemblance with UK, purified plasminogen (100 µg/ml) and t-PA (100 ng/ml) gave responses that were not any higher than the background of the assay.

Reproducibility. The reproducibility of the assay was estimated using a sample of pooled normal plasma and of urinary UK. The samples were analyzed 5 times (plasma) and 8 times (UK) in a single assay and intra-assay variations of, respectively, 3% (SD) and 6% (SD) were found. The same samples gave in 6, respectively, 8 consecutive assays inter-assay variations of 22% (SD) for the plasma and 18% (SD) for the urinary UK.

Efficiency in plasma and culture media. In human plasma and culture media, where many other proteins are present, the binding of UK to the coated microtiterplates could occur less efficiently as in buffer. In Fig. 1 it is shown that the dose(log scale)-response curves of urinary UK in buffer and in immunodepleted plasma (A) as well as those of purified pro-UK from monkey kidney in buffer and in depleted culture medium (B) each coincide over two decades of concentration. This indicates that UK is measured equally efficiently in culture media, plasma and buffer.

Comparison of different forms of UK. Generally, UK may occur in three forms: a zymogen, an active enzyme and an enzyme-inhibitor complex. In Fig. 1 it is shown that the dose-response curves of DFP-pretreated urinary UK and untreated UK in immunodepleted plasma (A) as well as those of purified pro UK from monkey kidney and the herefrom obtained active UK in buffer (B) each

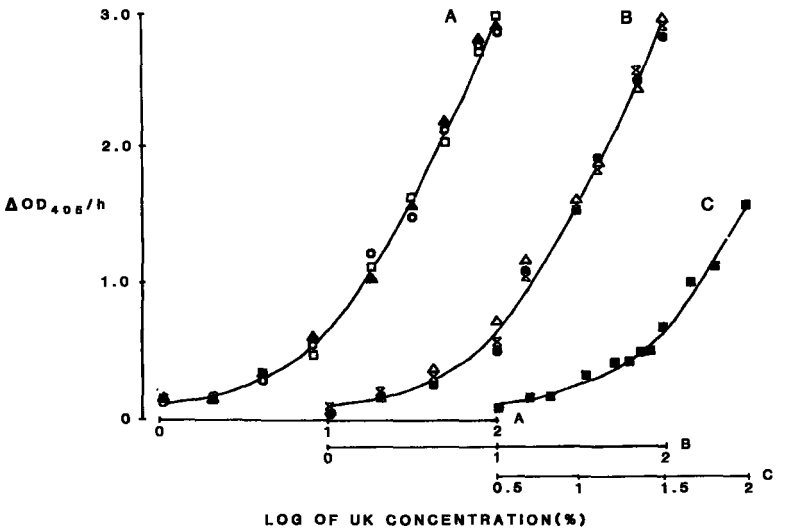

FIG. 1

Comparison of dose(log scale)-response curves of various UK's, measured under different conditions with the ELISA for UK:

A. Urinary UK: curves were obtained by serial dilutions of DFP-pretreated Choay UK in an excess of immunodepleted plasma (o), and of untreated Choay UK in Buffer A (▲) or in an excess of immunodepleted plasma (□). (UK concentration of 10 ng/ml was arbitrarily set at 100%).

B. Monkey kidney UK: curves were obtained by serial dilution of pro-UK in an excess of immunodepleted medium (△), and of pro-UK (●) and UK (⊻) derived herefrom by previous incubation with plasmin, in Buffer A (UK concentration of 1 IU monkey kidney UK per ml was arbitrarily set at 100%).

C. Plasma UK: the curve was obtained by mixing immunodepleted plasma with untreated plasma in ratio's varying from 33 to 0 (■) (the UK concentration in the untreated plasma was arbitrarily set at 100%).

coincide over two decades of concentration. This illustrates that in (A) the UK-inhibitor complexes formed in the plasma from the untreated UK (99% of the activity had disappeared by the time the antigen was measured) are immunochemically indistinguishable from the DFP-pretreated UK and determined with the same efficiency, and that in (B) the same holds true for pro-UK and UK. From this we conclude that the assay measures the total UK antigen present, irrespective of its molecular form. In accord, the plasma UK antigen, which consists of different molecular forms (see ref. 6), gives a dose-response curve (C) running parallel to curves A and B.

APPLICATIONS

Quantitation in culture media. A few examples are given in which the advantages of the assay are demonstrated in practice. In Fig. 2 the rate of UK production by a monkey kidney cell culture is shown on consecutive days. The spontaneous activity of UK, measured on the synthetic substrate S-2444, decreases from day 2 to day 10. However, the rate of UK antigen production increases substantially over the same period of time, together with the activity on S-2444 induced by previous incubation of the samples with plasmin. This indicates that in reality the cells start producing UK antigen in the form of the plasmin activatable proenzyme pro-UK. Consequently, the spontaneous activity on S-2444 is not representative for the total UK production and is probably caused by spontaneous activation of pro-UK in the early stages of production.

In Fig. 3, curve A, the production of UK antigen by human endothelial cells is followed for 48 hrs. The cells are known to produce substantial amounts of t-PA inhibitor, which also blocks UK activity. Despite the impossibility to follow UK production by its activity, the production can now be recorded by antigen measurement. Curve B, obtained by growing the cells in the presence of the m-RNA synthesis inhibitor actinomycin D, shows no antigen production, indicating that the increase of UK antigen in A, indeed, is the result of protein synthesis by the cells.

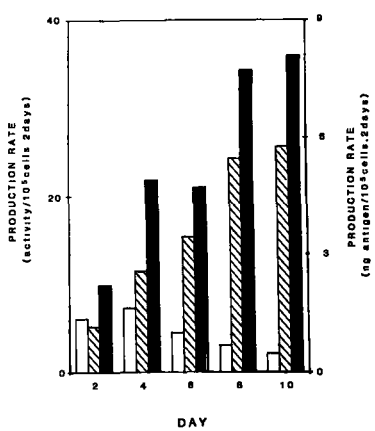

FIG. 2

Rate of UK production by a monkey kidney cell culture on consecutive days. The production rate of UK antigen (closed bars) was expressed as ng UK/10^5 cells.2 days, and for the spontaneous activity ($\Delta OD.10^3/h$) on S-2444 (open bars) or the activity on S-2444 induced by plasmin activation (hatched bars) as activity/10^5 cells.2 days.

In a primary culture of monkey kidney cells (18), culture fluid was replaced by serum-free maintenance medium M199. During a period of 10 days every two days 80% of the culture medium was harvested and replaced by fresh maintenance medium. UK antigen and activity were measured in these media as described in Materials and Methods. During this 10-day period the cell concentration declined from 0.84×10^6 cells/ml to 0.4×10^6 cells/ml.

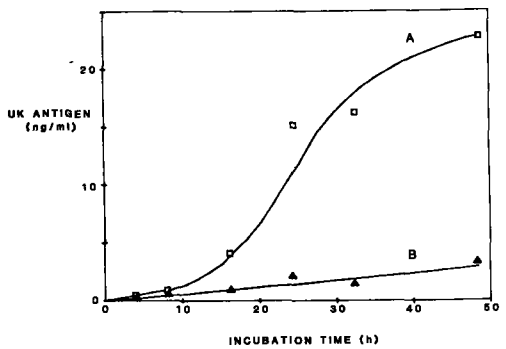

FIG. 3
UK antigen production by a human endothelial cell culture followed for 48 hours. Endothelial cells from aorta (third passage, see ref. 23) were grown without (A) and with actinomycin D (20 ng/ml) present (B) in the culture medium (M199 + 0.03% BSA). Samples from the medium were taken at the times indicated and UK antigen was determined as described in Materials and Methods. Cells grown with actinomycin D were viable at the end of the study.

In Table 1, some data are collected of UK antigen measurements in culture media without active UK. The results show values varying from 0 ng/ml for Bowes melanoma cell culture medium to 65 ng/ml for the monkey kidney cell culture medium. The production of 23 ng/ml by the endothelial cells is substantial and makes the endothelial cells of the vascular wall a possible candidate for the production of the urokinase occurring in the blood.

TABLE 1

UK Antigen, Determined with the ELISA in Culture Media without Active UK

Culture medium	UK antigen (ng/ml)
Bowes melanoma cells	0
Human hepatocytes	0.25
Hep G2 cells	0.5
Human skin fibroblasts	1.2
Human endothelial cells from aorta	23
Monkey kidney cells	65

Quantitation in human plasma. In a group of 54 normal, healthy individuals the arithmetrical mean of the UK antigen was found to be 3.5 ± 1.4 ng/ml, consistent with 3.2 ng/ml found for the pool. The SD of 1.4 ng/ml indicates a substantial variation between the individual levels; for, the method as such is far more sensitive with an interassay variation of the pool of only 20% or 0.7 ng/ml (see above, reproducibility). In Table 2 it can be seen that the distribution of the individual levels is asymmetric. The asymmetric distribution and relatively large variation are probably caused by the fact that the ELISA detects the sum of the three different molecular forms of UK occurring in human plasma (proenzyme, active enzyme and enzyme inhibitor complex, see ref. 6), which each may vary through the individuals. For the same reason, the individual UK activities measured after activation of pro-UK by dextransulphate euglobulin fractionation of the plasma, do not

correlate very well with the antigen found (not shown). In our experience with the ELISA thusfar (clinical studies, n = 300), 1.2 ng/ml UK is the lowest level found. This is far enough above the detection limit of the method (0.1 ng/ml, see above) to be estimated with accuracy.

TABLE 2

Distribution of UK Antigen in Plasma of 54 Normal, Healthy Individuals

UK Antigen (ng/ml)	n
1.4 - 2.1	7
2.1 - 2.7	8
2.7 - 3.3	14
3.3 - 4.0	7
4.0 - 4.6	9
4.6 - 5.3	3
5.3 - 5.9	2
> 5.9	4

Reference to other published UK-antigen assays

Several immunological methods for estimation of UK antigen levels have been described in the last few years (5,11-14). The most sensitive of these is a competitive radioimmunoassay (13) with a detection limit of 2×10^{-16} mol UK. The present work describes the development of an ELISA in which polyclonal affino-purified anti-UK antibodies are used. The detection limit of this assay amounts 3×10^{-16} mol UK, which is sensitive enough to determine the UK concentration in human plasma and in cell culture media. The assay is about 10 times more sensitive than the EIA described by Hérion et al. (14), who used monoclonal antibodies. It turns out to be specific for UK without any interference by t-PA or other plasma proteins. Since ELISA techniques, needing no special facilities for radioactive work, are applicable on a larger scale than RIA's, we consider this assay to be a good alternative for the RIA published by Huber et al. (13).

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CHAPTER III

SENSITIVE BIOLOGICAL IMMUNOASSAY FOR THE DETECTION OF SINGLE-CHAIN- (scu-PA) AND TWO-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (tcu-PA) IN PLASMA

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SUMMARY

Two kinds of biological immunoassay (BIA) were developed for the measurement of the active, two-chain- and the inactive, single-chain form of urokinase-type plasminogen activator (tcu-PA and scu-PA). In both cases u-PA was immuno-immobilized to wells of microtiterplates coated with antibodies raised against urinary u-PA. Subsequently the activity of the immobilized antigen was detected; in one set-up with the synthetic substrate for tcu-PA, S-2444, and in the other with the natural substrate plasminogen and the synthetic substrate for plasmin, S-2251. With both methods tcu-PA activity could be detected and in the latter method activity could be inhibited by adding anti-u-PA to the plasminogen/S-2251 mixture. scu-PA showed a detectable response after pre-activation of the immuno-immobilized material with plasmin. This made it possible to determine the separate levels of both molecular forms of u-PA. Detection of activity with plasminogen/S-2251 appeared to be the more sensitive method (lower limit of sensitivity 0.01 IU/ml) and the measurement of activity could be performed within a few hours. This set-up had intra- and inter-assay coefficients of variation of 4% and 10%, respectively. The method was applied to determine the low endogenous concentration of scu-PA and tcu-PA in plasma samples. In the plasma of normal healthy subjects (n=52) no detectable tcu-PA was found, and the scu-PA level was 0.21 ± 0.04 IU/ml (mean ± SD) with a Gaussian distribution. In a group of patients with liver insufficiency (n=23) and the few nephrectomized patients studied (n=3), significantly enhanced levels of scu-PA were found (0.41 ± 0.12 and 0.36 ± 0.10 IU/ml, respectively; $P<0.001$). In these plasmas tcu-PA was also detected (0.21 ± 0.16 and 0.10 ± 0.03 IU/ml, respectively; $P<0.001$).

INTRODUCTION

Urokinase-type plasminogen activator (u-PA), a serine protease originally recovered from urine (1), is able to promote the dissolution of fibrin clots by converting the zymogen plasminogen to active plasmin. It is one of the two main types of plasminogen activator that can be found in mammals. The other type, designated tissue-type plasminogen activator (t-PA) differs in molecular weight,

immunological reactivity and amino acid sequence (2, 3).

The molecular form of u-PA detected originally is an active M_r 55,000 molecule that consists of two disulfide-bridged polypeptide chains of, respectively, 33 and 22 kDa with the active centre at the 33 kDa chain (4, 5). Urokinase-type plasminogen activator also occurs in an inactive, single-chain form (scu-PA) (6-9). Proteolytic cleavage by plasmin converts scu-PA to the active, two-chain molecule (tcu-PA) (6, 8-12).

u-PA can also be recovered from various culture media (13, 14) and from plasma (15-17). In plasma taken from a person at rest, u-PA constitutes about 50% of the potential fibrinolytic activity (18) and recently it has been found that after certain stimuli such as exercise or DDAVP infusion there is an enhancement in plasma of the plasma u-PA level concurrent with the long-known enhancement of t-PA (19, 20). This appears to be of importance as the mechanisms of clot lysis by u-PA and t-PA are complementary (21). Furthermore, the combination of u-PA and t-PA may act synergistically in thrombolysis (22-25). These findings indicate the physiological significance of plasma u-PA for blood fibrinolysis.

To facilitate advances in physiological studies of plasma u-PA, specific and sensitive immuno-assays have been developed (16, 26-33). In a previous paper we have described an enzyme-linked immunosorbent assay (ELISA), based on polyclonal antibodies (34), that measures u-PA antigen irrespective of its molecular form (scu-PA, tcu-PA or tcu-PA in complex with inhibitors). In order to determine levels of active enzymes, biological immunoassays (BIA) have been described in which the activity of the immuno-immobilized material is measured (35, 36). Such assays have also been developed for u-PA (37-41), but these are either not able to measure the low levels of u-PA (nanograms per millilitre) normally present in plasma (37, 41) or do not discriminate between tcu-PA and scu-PA (38-40). Using the same immuno-immobilization procedure as described earlier for the u-PA ELISA, we developed a BIA which may distinguish between the active, two-chain form and the inactive, single chain form of u-PA and is sensitive enough for measurement of the endogenous u-PA level in plasma. Part of these results have been presented before (42, 43).

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: Pyro-Glu-Gly-Arg-pNA (S-2444), H-D-Val-Leu-Lys-pNA (S-2251) and human plasmin from KabiVitrum, Stockholm, Sweden. Aprotinin (Trasylol) from Bayer, Leverkusen FRG. Urinary urokinase from Choay, Paris, France. Human plasminogen and CNBr-fragments of fibrinogen were kindly provided by respectively, Drs D W Traas and W Nieuwenhuizen from our Institute.

Scu-PA (M_r 55,000) was from a human fibroblast cell culture medium (44).
Rabbit anti-u-PA IgGs isolated from antisera raised against urinary u-PA were obtained as described previously (15).

Platelet-poor citrated plasma was prepared as described by Kluft et al. (45).

Urokinase-depleted plasma and immunodepleted culture medium was prepared by immunoadsorption with immobilized anti-u-PA IgGs as described previously (18).

Patient plasmas were kindly provided by Dr E J P Brommer (46, 47).

BIA for u-PA

Immuno-immobilization of u-PA. u-PA was immuno-immobilized to the wells of a PVC microtiterplate coated with antibodies raised against u-PA, essentially as described in ref. 34 for the ELISA of u-PA. Sample dilutions of 150 µl in 0.01 M sodium phosphate buffer pH 7.4 containing 0.14 M NaCl, 5 mM EDTA, 1 mg/ml bovine albumin, 0.01% (v/v) Tween 80 and 0.02% (m/v) NaN_3 (EDTA buffer), were incubated overnight. Subsequently the wells were washed three times with 0.01 M sodium phosphate buffer pH 7.4 containing 0.14 M NaCl, 0.01% (v/v) Tween 80 and 0.02% (m/v) NaN_3 (PBS-Tween).

Pre-activation of scu-PA. Routinely the tcu-PA activity and the sum of the activities of plasmin-activatable scu-PA and tcu-PA were determined in parallel wells, the latter portion after a pre-activation step with human plasmin. The latter was achieved by incubating the immuno-immobilized material 30 min at room temperature with 1 mU/ml plasmin in EDTA buffer, which was sufficient for complete conversion of scu-PA to active tcu-PA (44). After this treatment the wash cycle was repeated, as described above.

Detection of tcu-PA activity.

S-2444: To detect activity, wells were incubated, with the microtiterplates in sealed plastic bags, at 37 °C with 150 µl 0.1 M Tris/HCl, 0.1 M NaCl, pH 8.8, containing 0.1% Tween-80, 60 KIU/ml aprotinin and 0.55 mM S-2444. The production of p-nitroaniline (pNA) was followed discontinuously at 405 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Irvine, Scotland) during at least 24 hours. The response was calculated from the linear part of the curve and expressed as the change in absorbance per hour.

Plasminogen/S-2251: The activity with plasminogen and S-2251 was determined at 25 °C in a volume of 150 µl, essentially as described by Verheijen et al. (48) for the measurement of tissue-type plasminogen activator in a soluble system. Specificity of the observed response was ascertained by the addition of anti-u-PA (1 µg/ml) to parallel sample wells. The production of pNA was followed during a period of four hours, at 405 nm with a Titertek Multiskan spectrophotometer. The response was calculated from the linear part of the absorption versus t^2 curve and was expressed as the change in absorbance per square hour.

Calculation of activity: Activities were assessed by reading the responses from calibration curves obtained by serial dilution of pooled plasma in EDTA buffer that had been pre-activated with plasmin as described above. Calibration of u-PA in plasma was carried out with the WHO International Standard for u-PA (batch nr 66/46, Natl. Inst. Biol. Stand. and Control, London, UK).

Statistical analysis: Results are expressed as mean ± SD. Statistical analysis was performed using the paired t-test.

RESULTS AND DISCUSSION

Comparison of methods and preference

In Fig. 1 it is shown that the activity of tcu-PA immuno-immobilized to wells of a microtiterplate coated with anti-u-PA, could be detected with the synthetic u-PA substrate, S-2444 (solid line). A dose-dependent increase in response was obtained. A similar result was obtained when the activity was detected through activation of plasminogen and the subsequent cleavage of the plasmin substrate S-2251 (broken line). The sensitivity of the latter method is much better: the intercept on the ordinate in Fig. 1 would correspond to a dose that is at least ten times lower than with S-2444. Contrary to the response measured on S-2444, the response on plasminogen/S-2251 could be fully inhibited by adding anti-u-PA to the assay mixture (compare encircled symbols). Apparently the synthetic substrate for u-PA, S-2444, is much less susceptible to steric hindrance caused by the antibodies than the natural substrate plasminogen.

scu-PA showed no response, neither on S-2444 (Fig. 2A: open triangles) nor on plasminogen/S-2251 (Fig. 2A: open circles), but a dose-dependent response was obtained after pre-activation of the immobilized material with plasmin (Fig. 2A:

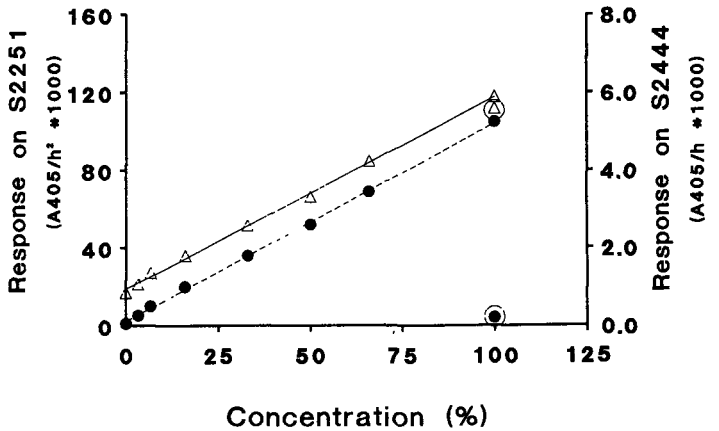
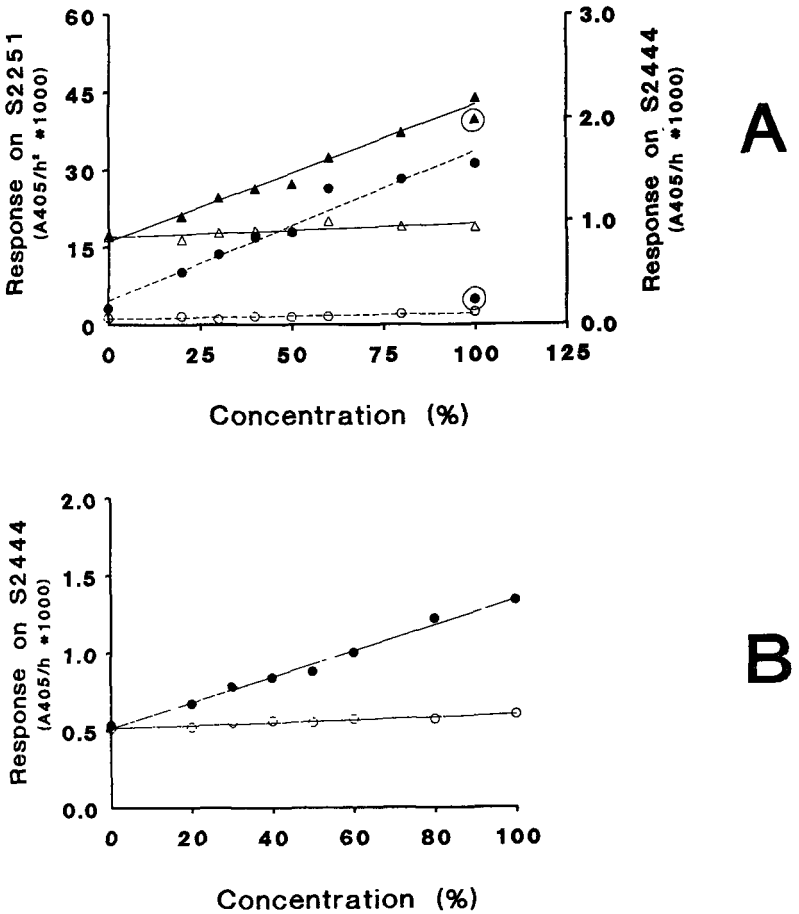


Figure 1. Response of immuno-immobilized tcu-PA from urinary source on S-2444 (solid line) and on plasminogen/S-2251 (broken line). tcu-PA was serially diluted and bound to the wells of a microtiterplate coated with anti-u-PA. Response was detected as described in Materials and Methods. (A concentration of 0.23 IU/ml was arbitrarily set at 100%). Encircled symbols show the effect of the addition of anti-u-PA (1 μg/ml, final concentration) to the assay mixture. Lines drawn are regression lines.

closed triangles, closed circles). Even after the 4-hour assay period with plasminogen/S-2251 the not pre-activated scu-PA (Fig. 2A, open circles) had not become activated. This is shown in Fig. 2B where subsequently the response on S-2444 was determined without any further pre-activation. Only the material described in Fig. 2A that had already been pretreated with plasmin (closed circles) showed a response in Fig. 2B. The untreated material (open circles) could still be activated though (not shown).

The above results confirm the previously reported finding of Corti et al. (37) that it is possible to distinguish between immuno-immobilized tcu-PA and scu-PA with the S-2444 method and, furthermore, demonstrate that this is also possible with the plasminogen/S-2251 method.

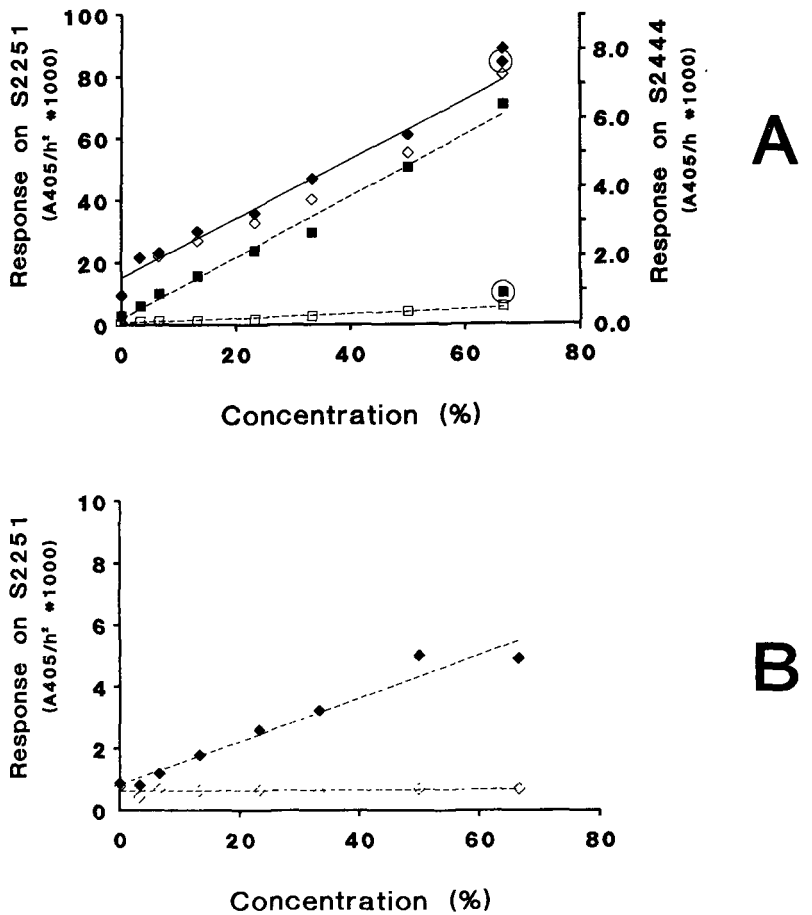
The two methods were applied to the u-PA present in plasma. With plasminogen/S-2251 (Fig. 3A: broken lines) a dose-dependent response was obtained only after pre-activation with plasmin (closed squares), indicating that the zymogen scu-PA but no active tcu-PA is present in plasma. This was confirmed in another study where u-PA from plasma was first purified and then measured with the assay (49).



Figures 2 A and B. Response of immuno-immobilized scu-PA from human fibroblast cell culture medium, on S-2444 (solid lines) and on plasminogen/S-2251 (broken lines). The scu-PA containing medium was serially diluted and the scu-PA was bound to the wells of a microtiterplate coated with anti-u-PA. Response was detected as described in Materials and Methods. (A concentration of 0.07 IU/ml was arbitrarily set at 100%). Encircled symbols show the effect of the addition of anti-u-PA (1 μg/ml, final concentration) to the assay mixture. Lines drawn are regression lines.

A and B: Response without (▵ , o) and with plasmin pre-activation (▲ , ●).

B: Measurement of the response on plasminogen/S-2251 in A was followed, after washing away the plasminogen/S-2251, by measurement of the response on S-2444 (without any further plasmin pre-activation).



Figures 3 A and B. Response of immuno-immobilized plasma u-PA on S-2444 (solid line) and on plasminogen/S-2251 (broken lines). Curves were obtained with serial dilutions of plasma (diamonds, squares); 100% concentration signifies undiluted plasma. Response was detected as described in Materials and Methods. Encircled symbols show the effect of the addition of anti-u-PA (1 μg/ml, final concentration) to the assay mixture. Lines drawn are regression lines.

A and B: Response without (◇ , □) and with plasmin pre-activation (◆ , ■).

B: Measurement of the response on S-2444 in A was followed, after washing away the S-2444, by measurement of the response with plasminogen/S-2251 (without any further plasmin pre-activation).

Surprisingly, when detection occurred with S-2444, the u-PA present in the plasma was already active (open diamonds, Fig. 3A) and pre-activation with plasmin had no additional effect (closed diamonds, Fig. 3A). However, when the response on plasminogen/S-2251 was subsequently determined without any further pre-activation (Figure 3B), the material that already had been pretreated with plasmin (closed diamonds) showed a response but the untreated material (open diamonds) did not. At present we have no explanation for this discrepancy between the activity measurement of plasma u-PA on the synthetic substrate S-2444 and the natural substrate plasminogen. Studies with scu-PA purified from plasma (49) showed a normal behaviour on S-2444, similar to that reported by Corti et al. (37), Günzler et al. (41) and in this study (Fig. 2A, triangles) for scu-PA from other sources.

Comparison of the results obtained with both methods (Figures 1A, 2A and 3A) shows that: 1. the lower limit of sensitivity of the plasminogen/S-2251 method is at least ten times lower than that of the S-2444 method; 2. the nature of the observed response with plasminogen/S-2251 can be ascertained by the inhibitory effect of anti-u-PA and 3. with the plasminogen/S-2251 method measurement of activity could be performed within a few hours versus 24 hours with the S-2444 method. On top of that we are left with the unexplained effect of plasma u-PA on S-2444 in Fig. 3A. Based on these observations we consider the plasminogen/S-2251 assay the method of preference for the detection of scu-PA and tcu-PA in plasma.

Validation of plasminogen/S-2251 assay

Background and detection limit. The background response in plasma or culture media immunodepleted in u-PA was equal to that in buffer, about 0.003 A405/h^2. For plasmin-treated samples it was somewhat higher, about 0.005 A405/h^2. The detection limit of the assay defined according to Rodbard (50) as the smallest response statistically significantly different from the background, was 0.010 A/h^2 for duplicate measurements. This corresponds with 0.01 IU/ml u-PA.

Reproducibility and efficiency. The inter- and intra-assay variations obtained with tcu-PA and plasma u-PA (n=10) were 10% and 4%, respectively. The dose-response curves (response, detected after plasmin treatment) of scu-PA added to immunode-

pleted plasma or buffer coincided (not shown), which indicates that the recovery of activity in plasma is about 100% and shows that the assay is equally efficient in buffer and in plasma.

Application

In a group of healthy subjects (n=52) tcu-PA was below the lower limit of sensitivity of the assay (≤ 0.01 IU/ml). As can be seen in Fig. 4 the scu-PA levels showed a Gaussian distribution with a small standard deviation (0.21 ± 0.04 IU/ml). With a specific activity of u-PA of 100,000 IU/mg (51) the mean level would correspond to 2.1 ng/ml of scu-PA.

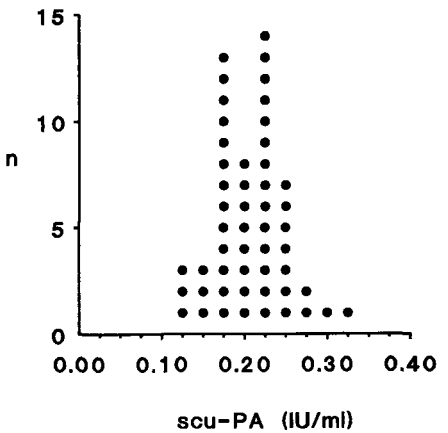


Figure 4. Distribution of scu-PA levels in plasma of 52 healthy subjects.
Detection method: plasminogen/S-2251.

In a group of patients with liver insufficiency (n=23) and the few nephrectomized patients studied (n=3) significantly enhanced levels of scu-PA were found (0.41 ± 0.12 and 0.36 ± 0.10 IU/ml, respectively; P≤0.001). In plasmas of these patients tcu-PA was also detected (0.21 ± 0.16 resp. 0.10 ± 0.03 IU/ml; P≤0.001). The enhanced level in the nephrectomized patients might indicate that the release of urokinase to the blood is not solely dependent upon kidneys. The enhanced level in the patients with liver insufficiency might indicate an impaired clearance of u-PA from the blood or an enhanced synthesis of u-PA by the liver.

Comparison with published assays

Immuno-immobilization of u-PA-antigen followed by detection of the activity of the bound material has been described by others (37-41). Our assay distinguishes between active tcu-PA and inactive scu-PA and has a detection limit of 0.01 IU/ml at least four times more sensitive than the hitherto reported assays. Assays with S-2444 as described by Corti et al (37) also distinguish between tcu-PA and scu-PA but are far more insensitive (lower limit of sensitivity 2.5 IU/ml) and therefore not suitable for measurement of endogenous scu-PA levels in plasma.

The measurement of tcu-PA and scu-PA levels with our assay, in combination with the measurement of the total u-PA-antigen (see ref. 34, our previously described ELISA) provides the opportunity to assess the amount of u-PA antigen not activatable by plasmin. In pooled plasma total u-PA antigen was found to be 3.3 ng/ml, scu-PA 2.1 ng/ml and tcu-PA <0.1 ng/ml, which implies that 1.1 ng/ml is inactive and probably represents u-PA in complex with inhibitors or scu-PA inactivated by thrombin or elastase (12, 52-54).

The combination of BIA and ELISA makes it possible to monitor scu-PA conversion in clinical studies.

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CHAPTER IV

CHARACTERIZATION OF SINGLE-CHAIN- (scu-PA) AND TWO-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (tcu-PA) FROM HUMAN PLASMA: MOLECULAR FORMS AND FIBRIN AFFINITY

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Submitted for publication.

SUMMARY

The different molecular forms of urokinase-type plasminogen activator (u-PA) present in human plasma were separated and characterized. On gelchromatography plasma was shown to contain the plasmin-activatable zymogen, single-chain u-PA (scu-PA), at M_r 55,000 (two-third of total u-PA antigen) and inactive u-PA-related protein, probably two-chain u-PA (tcu-PA) irreversibly bound to an inhibitor, of higher molecular weight (one-third of total u-PA antigen). Separation was also carried out with plasma that had previously been activated by dextran sulphate (DXS) treatment. Apart from aggregated and largely inactive u-PA antigen eluting in the breakthrough of the gelchromatography column, a single peak of u-PA activity was found, but at a M_r around 150,000, much higher than that of scu-PA. SDS-PAGE followed by zymography showed that the activity of the 150 kDa peak resided on a 55 kDa polypeptide which could be inhibited by anti-u-PA antibodies and most likely was tcu-PA.

The u-PA antigen and activity of the 150 kDa peak showed affinity for fibrin/Celite and for forming fibrin clots. No such affinity was observed with 55 kDa scu-PA from human plasma or with 55 kDa tcu-PA from urine. When, however, increasing amounts of tcu-PA from urine were added to DXS-treated plasma they also proportionally bound to forming fibrin clots. The results indicate that the observed fibrin affinity of the 150 kDa peak is not an intrinsic property of active 55 kDa tcu-PA, but is brought about by complexation with another plasma component, and that activation of 55 kDa scu-PA in plasma is accompanied by this complexation. We suggest that in the circulation the fibrin-bound 150 kDa complex restricts plasminogen activation by u-PA to the fibrin clot.

INTRODUCTION

The lysis of fibrin in the circulation is catalyzed by the enzyme plasmin, which is formed from the inactive zymogen plasminogen through limited proteolysis by plasminogen activators (PAs) (1, 2). Two pathways of plasminogen activation may be distinguished: the extrinsic and the intrinsic pathway (3). The PA of the extrinsic system is designated as tissue-type plasminogen activator (t-PA) (4). In response to

stimuli such as exercise or venous occlusion, active t-PA is released from the endothelial cells of the vessel wall into the blood stream (3, 4). The intrinsic pathway comprises activators circulating in the blood as inactive proenzymes, which in vitro can be activated by treatment of plasma with dextran sulphate (DXS) after removal of inhibitors by iso-electric precipitation (3). Part of the potential intrinsic activity is indeed brought about by a plasminogen proactivator which is dependent on the contact system for activation (5). This PA was recently identified as a M_r 600,000 molecule with the active centre on a 110 kDa polypeptide. Upon contact activation the 110 kDa polypeptide is converted from an inactive, single-chain- into an active, cleaved, disulphide-bridged polypeptide (6).

Another part of the potential intrinsic activity comes from urokinase-type plasminogen activator (u-PA), which in vitro also becomes activated by the DXS treatment of plasma (7-9). Urokinase is a plasminogen activator originally recovered from urine and from culture media of kidney cells (10, 11), and has more recently been shown to be present in the blood (12-14). The main molecular forms of urokinase found in urine were active, two-chain polypeptides (tcu-PA) with molecular weights of about 55,000 and 33,000 (15, 16). In culture media and urine also inactive urokinase forms were also found which could be activated by plasmin or trypsin (11, 17), and later were proven to be single-chain polypeptides (scu-PA) (18-23).

In the present study we characterized the different molecular forms of u-PA present in human blood. We also investigated the affinity for fibrin, since it was already reported that the extrinsic activator, t-PA, preferentially binds to fibrin (24, 25) as may some of the u-PA forms (21, 22, 26-28), inducing fibrin-selective clot lysis (26, 29, 30).

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: Dextran sulphate (M_r 500,000), protein A-Sepharose, Ultrogel ACA 34 and low molecular weight markers for electrophoresis from Pharmacia/LKB, Uppsala, Sweden. Flufenamic acid and benzamidine hydrochloride hydrate from Aldrich-Europe, Beerse, Belgium. Ammonium sulphate p.a. and molecular weight markers (MS-11) for gel chromatography from Serva, Heidelberg, Germany. S-2251 from KabiVitrum, Stockholm, Sweden. Thrombin (bovine, 5,000 NIH units per tablet) from Leo Pharmaceutical Industries, Ballerup, Denmark. Aprotinin (Trasylol) from Bayer, Leverkusen,

Germany. Celite (Hyflo-Supercel) from Fisher Scientific, USA. Urinary tcu-PA was from Leo Pharmaceuticals Industries and from Choay, Paris, France. Human plasmin, plasminogen, t-PA and rabbit anti-t-PA IgGs were kindly provided by Drs D W Traas and J H Verheijen from our Institute.

Plasminogen-rich bovine fibrinogen was prepared according to Brakman (31).

Rabbit and goat anti-u-PA IgGs were isolated from antisera raised against urinary u-PA as described (12).

Platelet-poor citrated plasma was prepared as described by Kluft et al. (32).

u-PA-depleted plasma was prepared by immunoadsorption with immobilized anti-u-PA IgGs as described previously (8).

Fractionation of plasma

51 and 90 ml of plasma were fractionated in essence as described in ref. 6, Scheme I, for Preparations I and III, respectively. In brief: in one procedure the fractionation was carried out in the presence of the protease inhibitors aprotinin (15 KIU/ml) and benzamidine (10 mM) to prevent activation (cf. Prepn III in ref. 6). In the other procedure, after the removal of inhibitors by iso-electric precipitation, treatment with dextran sulphate (30 μg/ml plasma) was included to accomplish activation (cf. Prepn I in ref. 6). Subsequently, the preparations were fractionated as described in ref. 6, Scheme II, and subjected to gelchromatography on a 100 x 2 cm Ultrogel ACA 34 column equilibrated with phosphate-buffered 1M NaCl, pH 7.4, containing 10 mM benzamidine in the case of Prepn III. Fractions of 2 ml were collected. Columns were, in separate runs, calibrated with molecular weight markers.

SDS-PAGE and zymography

SDS-PAGE was carried out on 14.5 x 16 x 0.1 cm slab gels according to Laemmli (33) with a 3.5% stacking gel and a 8% running gel. Non-reduced samples (25 μl) mixed with an equal volume of sample buffer, were applied to the slab gel and electrophoresed at 40 mA. Molecular weight calibration was performed using low molecular weight markers run in separate lanes. After completion of electrophoresis, zymography on plasminogen-rich fibrin-agarose underlays was performed in essence according to Granelli-Piperno and Reich (34). In parallel experiments appropriate amounts (30 μg/ml, ref. 35) of goat anti-u-PA IgGs were incorporated in the underlay to inhibit the activity of u-PA.

Assays

ELISA for u-PA antigen. u-PA antigen was detected with the ELISA described before (36).

Biological immunoassay (BIA) for tcu-PA and scu-PA (9, 37, 38). u-PA-antigen was immuno-immobilized to wells of polyvinylchloride microtiterplates coated with anti-u-PA IgG as described for the ELISA (36). tcu-PA activity was detected with plasminogen and S-2251, in essence as described by Verheijen et al. (39) for the measurement of tissue-type plasminogen activator in a soluble system. The sum of scu-PA and tcu-PA was detected after pretreatment of the immobilized material for half an hour at room temperature with plasmin (0.6 nM). Responses in BIA and ELISA were correlated via calibration with scu-PA purified from human fibroblasts, and calibration of u-PA antigen occurred as described (36). The lower limit of sensitivity of these assays is 0.1 ng/ml and the intra-assay variation 4%.

Fibrin plate assay. Plasminogen activator activity of samples was determined with the standardized fibrin plate assay (40). u-PA activity was determined as the difference of the plasminogen activator activity in the presence and absence of an excess of anti-u-PA IgGs. Activity in IU/ml was computed from the areas of lysed zones obtained in 17 h by comparison with the WHO International Standard for u-PA (code 66/46).

Assessment of fibrin affinity

Fibrin/Celite affinity chromatography. Fibrin/Celite chromatography was performed in essence as described by Husain et al. (41); untreated plasma (12 ml) or DXS-treated plasma (10 ml, see Fractionation of plasma) were applied at a rate of 15 ml/h to a silicone-treated glass column (1.5 x 25 cm) with fibrin/Celite packed to a volume of 16.5 ml.

Binding to forming fibrin clots. Clots were formed by mixing either 0.5 ml EDTA buffer (0.05 M sodium diethylbarbiturate pH 7.8, 0.10 M NaCl, 0.25% (w/v) gelatin, 2.7 mM ethylene diamine tetraacetate) containing an aliquot of purified tcu-PA and 1 mg/ml plasminogen-rich fibrinogen, or 0.5 ml of a double-concentrated DXS-treated plasma (see Fractionation of plasma), with 0.33 ml of EDTA buffer containing 0.01 M $CaCl_2$ and 20 NIH units/ml of thrombin. The mixture was allowed to stand for 15 min at room temperature, followed by 5 min at 37 °C before removal of the clot by centrifugation. Clots were washed twice in 0.5 ml EDTA buffer, dried on a tissue and either placed on a fibrin plate to measure the fibrinolytic activity, or incubated overnight at 37 °C in a total volume of 0.83 ml EDTA buffer containing 1 μM plasminogen and 35 IU/ml of t-PA, to recover tcu-PA in solution. In parallel experiments 0.5 ml of the respective tcu-PA preparations were mixed with 0.33 ml of EDTA buffer without $CaCl_2$ and thrombin to assess the tcu-PA concentration before clotting.

RESULTS

Molecular forms

The different molecular forms of u-PA present in plasma were fractionated in the presence of protease inhibitors as described in Materials and Methods, and further separated on ACA 34 gelchromatography. Fig. 1A, open squares, shows an asymmetrical peak of u-PA antigen with the top around M_r 55,000. Upon SDS-PAGE followed by zymography on fibrin-agarose underlays (Fig. 2 lane 5) no lysis could be detected; neither could active tcu-PA be found with a biological immunoassay (Fig. 1A open circles), but after activation of the immuno-immobilized antigen in the assay by plasmin a nearly symmetrical peak around M_r 55,000 was found (Fig. 1A, closed circles). This peak covers about two-thirds of the total u-PA antigen in Fig. 1A and most likely originates from the zymogen scu-PA. The remainder of the u-PA antigen (about one-third of total antigen), could not be activated by plasmin and probably represents tcu-PA irreversibly bound to an inhibitor.

Separation was also carried out with plasma that had previously been activated by DXS treatment. u-PA antigen (Fig. 1B, open squares) was found in the void volume of the column with a shoulder around M_r 800,000, at M_r 150,000 and a minute amount at M_r 55,000. The material eluting around M_r 800,000 and 55,000 appeared to be inactive, was not activatable by plasmin (Fig. 1B, closed circles) and is probably in vitro generated tcu-PA that had reacted with the inhibitor $alpha_2$-macroglobulin which elutes around M_r 800,000, respectively scu-PA that had been inactivated (e.g by thrombin, see ref. 42-44). Activity (Fig. 1B, open circles) was found in the void volume of the column (minor amount) and around M_r 150,000. In both cases the activity resided on a 55 kDa polypeptide which could be inhibited by anti-u-PA antibodies (Fig. 2 lanes 1, 3 vs. 2, 4) and was most likely tcu-PA.

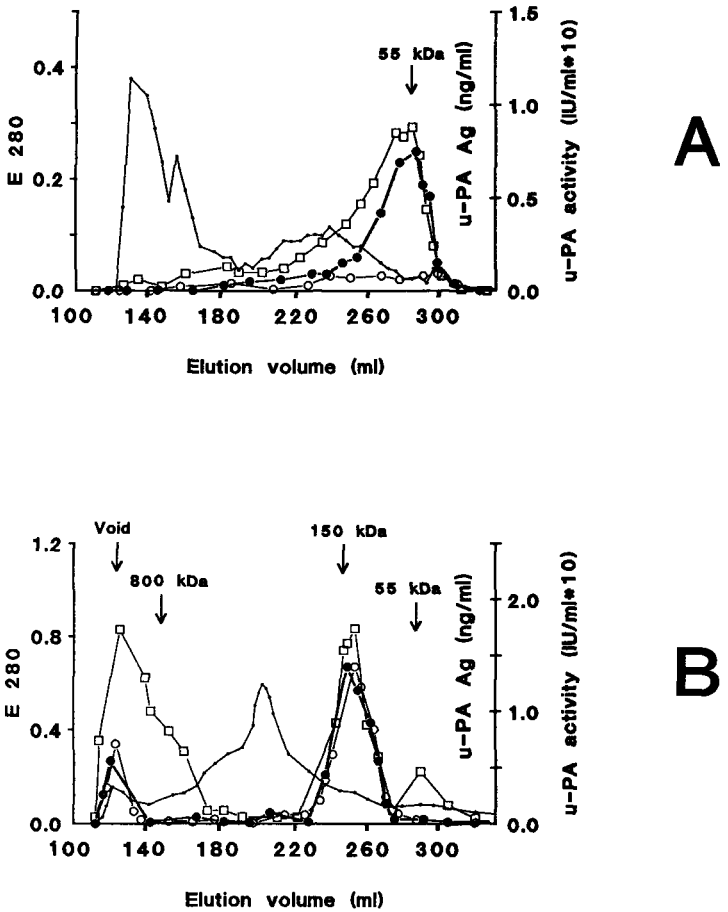

Figure 1. ACA 34 gelchromatography of plasmas, fractionated in the presence of protease inhibitors (A) and fractionated after DXS treatment (B). A and B: fractionation and subsequent ACA 34 gelchromatography in phosphate-buffered 1 M NaCl, pH 7.4, were performed as described in Materials and Methods. Elution profiles are shown of: u-PA antigen (□ - □); u-PA activity detected by BIA before (o - o) and after activation with plasmin (● - ●); E280,—.

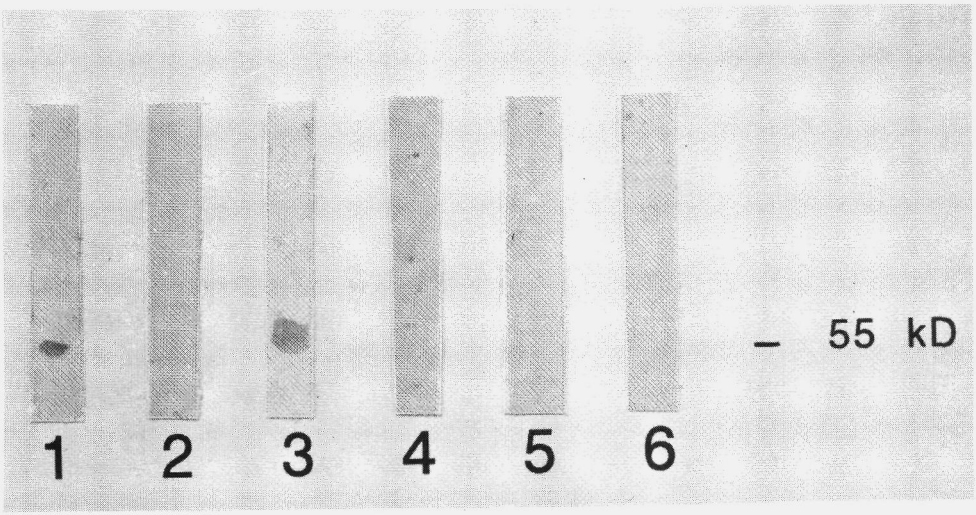

Figure 2. Zymography of peak fractions from Fig. 1A and B. SDS-PAGE of fractions was followed by zymography on fibrin-agarose underlays as described in Materials and Methods. Lanes 1, 2: void peak from Fig 1B; lanes 3, 4: 150 kDa peak from Fig. 1B; lanes 5, 6: 55 kDa peaks from Figs. 1A and 1B, respectively. To the underlays in lanes 2 and 4 anti-u-PA antibodies (30 μg/ml) were added.

Fibrin affinity

Fibrin affinity of the different molecular forms of u-PA in plasma was qualitatively assessed by fibrin/Celite affinity chromatography. Since this method involves preformed fibrin (41), the risk of scu-PA inactivation by thrombin (42-44) is avoided. With untreated plasma more than ninety percent of the plasmin-activatable scu-PA was present in the fall-through of the column, but with the DXS-treated plasma, which contains substantial amounts of active tcu-PA at M_r 150,000 (see Fig. 1B), more than ninety percent of the tcu-PA adhered to the column.

The apparent fibrin affinity of the 150 kDa plasma tcu-PA was quantified by binding to forming fibrin clots. Table I shows that with increasing amounts of the

purified 150 kDa tcu-PA a constant fraction of about twenty-five percent of the activity is missing in the fluid phase and that fibrinolytic activity of the clots dose-dependently increases. With 55 kDa urinary tcu-PA no such effects are seen.

Table I. Fibrin affinity of purified tcu-PA preparations.

Dose-dependent binding to forming fibrin clots was assessed as described in Materials and Methods. The binding was determined by measuring the concentration of tcu-PA in the fluid phase before clotting and after removal of the clot. tcu-PA concentrations were estimated with the fibrin-plate method and expressed in IU/ml (see Material and Methods). Fibrinolytic activity of clots was estimated by placing them on fibrin plates and measuring the area of the lysed zones obtained in 17 h.

Prepn.	tcu-PA activity in fluid phase (IU/ml)			fibrinolytic activity of clot (mm^2)
	before clotting	after removal of clot	% missing	
150 kDa plasma tcu-PA*				
	0.07	0.05	29	175
	0.09	0.06	33	226
	0.14	0.11	21	244
55 kDa urinary tcu-PA**				
	0.10	0.10	0	<10
	0.50	0.48	4	56

*: M_r 150,000 peak fraction from Fig. 1B.
**:commercially obtained, see Materials and Methods.

Quantification of fibrin affinity was also performed in plasma milieu. Fig. 3A shows that a similar result is obtained as found in the purified system of Table I: the dose-dependent increase in the fibrinolytic activity of the clots (closed circles) is parallelled by the disappearance of plasma tcu-PA activity from the fluid phase (inset, open portion of the bars). The experiment in Fig. 3A was repeated in Fig. 3B, but on this occasion the portions of plasma tcu-PA missing in the fluid phase were recovered from the fibrin clots and specifically measured by BIA. It is seen that at

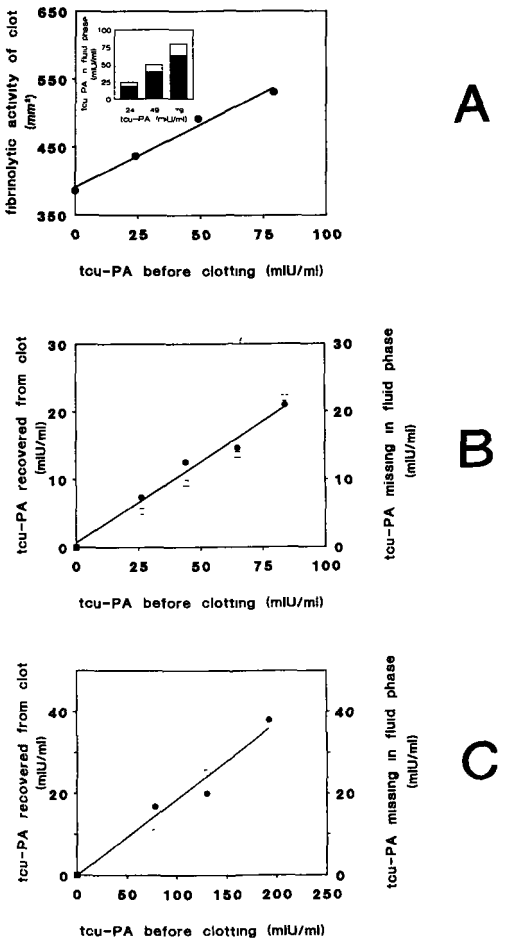

Figure 3. Fibrin affinity of tcu-PA in plasma milieu. Dose-dependent binding to forming fibrin clots was assessed as described in Materials and Methods. In A and B concentration ranges were obtained by mixing DXS-treated normal and u-PA depleted plasma. In C increasing amounts of 55 kDa urinary tcu-PA were added to DXS-treated, u-PA depleted plasma. In A (inset) total and black bars represent, respectively, the concentration of tcu-PA in the fluid phase before clotting and after removal of the clot. Open bars (A, inset) and open squares (B and C) represent the concentrations of tcu-PA missing in the fluid phase. tcu-PA concentrations were estimated with the fibrin plate method (A) or by BIA (B and C). In A fibrinolytic activity of clots (closed circles) was estimated by placing them on fibrin plates and measuring the area of the lysed zones obtained in 17 h. In B and C tcu-PA was recovered from the clot as described in Materials and Methods and subsequently measured by BIA (closed circles). Lines drawn represent regression curves.

all doses the missing (open squares) and recovered portions (closed circles) are almost equal, indicating full recovery of active tcu-PA. Fig. 3C shows that with 55 kDa urinary tcu-PA in plasma milieu a similar result was obtained as with plasma tcu-PA in Fig. 3B, despite the fact that 55 kDa tcu-PA in the purified system of Table I did not bind.

DISCUSSION

In this study the different molecular forms of u-PA present in plasma were investigated. In earlier reports from other research groups (13, 14) the occurrence of a u-PA-related 55 kDa polypeptide in plasma was already demonstrated, but in those studies no distinction was made between active tcu-PA and the zymogen scu-PA nor were the different molecular forms actually present in plasma before SDS treatment established. We subjected fractionated plasma to gelchromatography and demonstrated that it contains 67% plasmin-activatable scu-PA with a M_r of 55,000 and 33% inactive u-PA, not activatable by plasmin, of higher molecular weight. This corresponds well with our previous conclusion (45) that in plasma 2.1 ng/ml scu-PA next to 1.1 ng/ml inactive u-PA-inhibitor complex is present, but virtually no tcu-PA.

In plasma that previously had been activated by DXS, active tcu-PA was found, but almost no scu-PA. This is in line with previous reports indicating that in a DXS euglobulin fraction of plasma activators such as plasmin are present that may rapidly convert scu-PA into active tcu-PA (9, 46). The active tcu-PA was mainly found at M_r 150,000, a M_r much higher than that of scu-PA, or tcu-PA from other sources. SDS-PAGE followed by zymography (Fig. 2, lanes 1-4) demonstrated though that 55 kDa tcu-PA was involved, but probably bound to another plasma component, which made it elute at M_r 150,000. Self-aggregation of 55 kDa tcu-PA is considered unlikely since chromatography was performed in the presence of 1 M NaCl, under which condition tcu-PA from urine elutes at M_r 55,000 (not shown).

In our own earlier experiments (47) and those of others (48) it was observed that active tcu-PA present in DXS-treated plasma preferentially stuck to fibrin clots. In this study we further investigated the fibrin affinity, especially that of the active 150 kDa species. With fibrin/Celite affinity chromatography almost all of the active tcu-PA could be singled out from DXS-treated plasma, and in the experiments with

forming fibrin clots plasma tcu-PA was proportionally removed from the fluid phase and fully recovered, in active form, from the clots. The affinity of tcu-PA for fibrin was a special feature of the 150 kDa species from plasma and was not found with scu-PA from plasma (fibrin/Celite chromatography) or tcu-PA from urine (Table I). However, when DXS-treated, u-PA depleted plasma was reconstituted with 55 kDa urinary tcu-PA (Fig. 3C), the latter acquired fibrin affinity. This result suggests that the observed fibrin affinity of the 150 kDa species is not an intrinsic property of active 55 kDa tcu-PA, but is brought about by complexation with another plasma component. This is in agreement with the suggestion made by Kok and Nilsson (48) that "compounds other than fibrin and activators may be involved in the regulation of PA adsorption to fibrin in plasma".

Other investigators have reported on the presence in urine of u-PA with fibrin affinity (49-51). However, in those studies a 100 kDa protein was described which was a SDS-stable complex of 55 kDa tcu-PA with the inhibitor PAI-3 (51) with a specific activity much lower than that of 55 kDa tcu-PA. The specific activity of the 150 kDa species in this study calculated from the activity (BIA) and antigen concentration (ELISA) in Fig. 1B, was similar to that of the standard tcu-PA (about $1*10^5$ IU/mg, ref. 52).

The fibrin affinity of the 150 kDa species may be of physiological significance. In the circulation plasmin is generated by the action of the fibrin-bound extrinsic activator t-PA (25, 53, 54) and by the low activity of the intrinsic pro-activator scu-PA with respect to fibrin-bound plasminogen (30, 55, 56). The locally generated plasmin will initiate the reciprocal feed-back activation process of scu-PA and plasminogen. Owing to the formation of the 150 kDa complex described above, the generated 55 kDa tcu-PA may temporarily remain bound to the clot before its action is halted through protein inhibitors present in the blood. Consequently the plasminogen activation by u-PA in the circulation is restricted to the fibrin clot.

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CHAPTER V

AN ANALYSIS OF THE ACTIVATORS OF SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (scu-PA) IN THE DEXTRAN SULPHATE EUGLOBULIN FRACTION OF NORMAL PLASMA AND OF PLASMAS DEFICIENT IN FACTOR XII AND PREKALLIKREIN

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An Analysis of the Activators of Single-Chain Urokinase-Type Plasminogen Activator (scu-PA) in the Dextran Sulphate Euglobulin Fraction of Normal Plasma and of Plasmas Deficient in Factor XII and Prekallikrein

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Summary

An analysis was made of the various possible activators of single-chain urokinase-type plasminogen activator (scu-PA) in the dextran sulphate euglobulin fraction (DEF) of human plasma. scu-PA activators were detected in an assay system in which the substrate scu-PA, in physiological concentration (50 pM), was immuno-immobilized. After activation of the immobilized scu-PA for a certain period of time the activity of the generated amount of immuno-immobilized two-chain u-PA was determined with plasminogen and the chromogenic substrate S-2251. The scu-PA activator activity (scuPA-AA) in the DEF of plasmas deficient in factor XII or prekallikrein was about half of that in the DEF of normal plasma. Separation of scuPA-AA in the DEF by gel chromatography showed to major peaks, one eluting with an apparent M_r of 500,000 and the other around M_r 100,000. The former peak, which coincided with the activity peak of the kallikrein-kininogen complex, was absent in the DEF of plasma depleted of prekallikrein and therefore was identified as kallikrein. The latter peak was still present in the depleted plasma and most likely represents plasmin, because its scuPA-AA coincided with the activity peak of plasmin and could be fully inhibited by antibodies raised against human plasminogen. It is concluded that plasmin and the contact-activation factor kallikrein each contribute for about 50% to the scuPA-AA in the DEF. Compared on a molar basis, however, plasmin was found to be almost 1,000 times more effective than kallikrein, and we conclude, therefore, that in vivo plasmin is the primary activator of scu-PA and the role of the contact system is of secondary importance.

Introduction

Fibrinolysis is catalysed by the enzyme plasmin formed from the zymogen *plasminogen* by the specific action of plasminogen activators (1). Urokinase type plasminogen activator (u-PA) is a plasminogen activator which occurs in human plasma in a zymogen form (scu-PA, refs. 2–7). Proteolytic cleavage by plasmin converts the 55 kDa scu-PA into an active molecule with two disulphide linked polypeptide chains of respectively 33 and 22 kDa (8–11).

Ichinose et al. (12) investigated the activation of scu-PA by purified plasma proteins. They demonstrated that, besides plasmin, the contact activation factor kallikrein is also a most effective activator of scu-PA and postulated that contact system-dependent fibrinolysis would proceed via activation of scu-PA by kallikrein. Mides et al. (13, 14), Husveld et al. (15) and Hauert et al. (16) gave support for this idea by showing that in dextran sulphate (DXS)-treated fractions of plasma, kallikrein is the activator of scu-PA. Reports from our Institute have shown on the contrary, that contact activation stimulates fibrinolysis via activation of a novel, recently identified, plasminogen proactivator (17) and that kallikrein (and also factor XII_a) is not required at all for the activation of scu-PA (18–20). In the dextran sulphate euglobulin fraction (DEF) of plasmas deficient in factor XII or prekallikrein, fully-activated two-chain u-PA (tcu-PA) was still found. *In the* present study, using an assay system that specifically measures scu-PA activators, we re-investigated this matter, and made an analysis of the various possible scu-PA activators in the DEF and of their relative effectiveness. Part of these results have been presented before (21).

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Materials and Methods

The following materials were obtained from the indicated sources: Dextran sulphate (M_r 500,000), protein-A Sepharose 4B from Pharmacia, Uppsala, Sweden. Aprotinin (Trasylol®) from Bayer, Leverkusen, FRG. Ultrogel ACA 34 from L. K. B., Bromma, Sweden. Ammonium sulphate p.a. and molecular weight markers (*MS-11*) for gel chromatography from Serva, Heidelberg, FRG. Streptokinase (Kabikinase) and S-2251, H-D-Val-Leu-Lys-pNA, and human plasmin from KabiVitrum, Stockholm, Sweden. Chromozym PK, Bz-Pro-Phe-Arg-pNA.AcOH (PPAN), from Pentapharm A.G., Basle, Switzerland. GGACK, H-L-Glu-Gly-Arg-chloromethyl ketone, from Calbiochem, La Jolla, CA. Plasma kallikrein was obtained from Sigma Chemical Company, St Louis, MO. Human plasminogen, CNBr-fragments of fibrinogen and antibodies raised against plasminogen were kindly provided by respectively, Drs D. W. Traas, W. Nieuwenhuizen and D. C. Rijken from our Institute. Rabbit anti-u-PA IgGs isolated from antisera raised against urinary u-PA were obtained as described previously (2).

Platelet-poor citrated plasma and dextran sulphate euglobulin fraction (DEF) of plasma were prepared according to Kluft et al. (22, 23).

Fletcher and Hageman trait plasmas were obtained from George King, Overland Park, KS. A Hageman trait plasma, kindly provided by Dr. E. J. P. Brommer from our Institute, was artificially depleted of prekallikrein by immuno-adsorption with anti prekallikrein IgGs coupled to Sepharose-4B, in a similar manner to that previously described for the depletion of u-PA (18). After depletion, the concentration of prekallikrein was below the lower limit of sensitivity of the assay used (24). This implies that depletion occurred for at least 97%.

Gel Chromatography of DXS-Euglobulin Subfractions

DXS (30 μg/ml) was added to the supernatant of a euglobulin precipitate of 100 ml plasma as described by Kluft et al. (25). The precipitate so formed was centrifuged and dissolved in 0.14 M NaCl, 0.01 M sodium phosphate *pH 7.4 (PBS)* and subjected to ammonium sulphate fractionation to remove most of the fibrinogen. The fraction precipitating between 30% and 50% saturation was dissolved in PBS and subjected to protein-A Sepharose gel chromatography (1.5 × 2.5 cm column) to remove IgGs. In the resulting fall-through the NaCl concentra-

tion was raised from 0.14 M to 1 M This preparation was applied to a *100 × 2 cm Ultrogel ACA 34 column equilibrated* with phosphate buffered 1 M NaCl (pH 7.4), fractions of 1.9 ml were collected Plasma deficient in factor XII and depleted of prekallikrein (18 ml), was treated as above, *except that gel chromatography* was performed on a *90 ×* 1 2 cm Ultrogel ACA 34 column and fractions of 0 87 ml were collected

Assays

scu-PA activation As substrate scu-PA (M_r 55.000) from human fibroblast cell culture medium was used Prior to use, the medium was incubated for 30 min at 37° C with 9 mM diisopropylfluorophosphate and 500 KIU/ml aprotinin. Routinely scu-PA was immuno-immobilized to the wells of a PVC microtitre plate, essentially as described in Binnema et al (26) for the ELISA of u-PA, by an overnight incubation of 150 µl of the medium, diluted to a concentration of 50 pM scu-PA, in 0 01 M sodium phosphate buffer pH 7.4 containing 0.14 M NaCl, 5 mM EDTA, 1 mg/ml bovine albumin, 0 01% (v/v) Tween 80 and 0 02% (m/v) NaN_3 (EDTA buffer) plus 175 KIU/ml aprotinin. Subsequently, the wells were washed three times with *0 01* M sodium phosphate buffer pH 7 4 containing 0.14 M NaCl, 0 01% (v/v) Tween 80 and 0.02% (m/v) NaN_3 (PBS-Tween) The immobilized scu-PA was then incubated at room temperature, with samples of scu-PA activator (50 µl sample and 100 µl EDTA buffer) with or without aprotinin or anti-plasminogen as indicated in the legends of the figures. After an appropriate incubation period the scu-PA activator was washed off (five times with PBS-Tween) and the amount of immobilized tcu-PA was determined with plasminogen and S-2251, in essence as described by Verheijen et al (27) for the measurement of tissue-type plasminogen activator in a soluble system. In Table 1 the scu-PA activator activity (scuPA-AA) was calculated from the linear part of the time curve of the activation of scu-PA (cf. Fig 1) and is expressed as the percentage of immobilized scu-PA converted per minute scuPA-AA in the DXS-treated plasma fractions in Figs. 2 and 3 is expressed as the percentage of scu-PA that had been converted after a 1 h activation period.

Prior to determination of scuPA-AA, samples were immunodepleted of u-PA antigen to avoid possible interference by scu-PA or tcu-PA that a priori might have been present in the samples Immunodepletion was performed by incubation of the samples at room temperature for 2 h in wells of microtitre plates coated with antibodies raised against urinary urokinase as described for the ELISA of u-PA (26).

Kallikrein. Kallikrein activity was determined in essence according to Kluft et al. (24), using the chromogenic substrate for kallikrein, PPAN 10 µl samples were incubated with 200 µl Tris-imidazole buffer (µ = 0 15, pH 7.9) with 0.1% Carbowax and 40 µl PPAN (1 mM in distilled water) All incubations were performed at 37° C in microtitre plates The absorbance at 405 nm was recorded with a Titertek Multiskan spectrophotometer and the ΔOD/min was extrapolated from the linear part of the curve Activity was expressed as the change in absorbance per minute at 405 nm. Calibration was performed with pooled plasma after activation with DXS as described (24), and the kallikrein concentration was calculated from the activity assuming a plasma concentration of prekallikrein of 500 nM

Plasmin. Plasmin activity was assayed as described by Friberger et al (28). 10 µl samples were incubated with 190 µl 0.1 M Tris-HCl pH 7.5, 0.1% (v/v) Tween 80 and 10 µl S-2251 (10 mg/ml) at 25° C All incubations were performed in microtitre plates and the rate of release of para-nitroaniline (pNA) was determined at 405 nm with a Titertek Multiskan spectrophotometer. Calibration was performed with pooled plasma after activation with streptokinase as described (28), and the plasmin concentration was calculated from the activity assuming a plasma concentration of plasminogen of 2 µM.

Results

Activation of scu-PA by Plasmin

scu-PA, immuno-immobilized in physiological concentration (50 pM) to the wells of a microtitre plate, was treated with plasmin. Fig. 1 (closed symbols) shows that tcu-PA activity increased gradually with the activation time. With a concentration of 25 pM plasmin (closed squares), half that of the substrate scu-PA, in 2 h about one-third of the maximum tcu-PA activity was reached. Maximum tcu-PA activity was obtained in about 1 h with a ten-fold higher plasmin concentration (Fig. 1, closed triangles). Activation by plasmin was completely inhibited when aprotinin (open circles) or antibodies raised against plasminogen (open triangles) were present during activation. With buffer alone no activation occurred (open squares). The tcu-PA activity measured after activation could completely be inhibited by adding the specific synthetic inhibitor GGACK or antibodies raised against urinary urokinase to the reaction mixture (not shown).

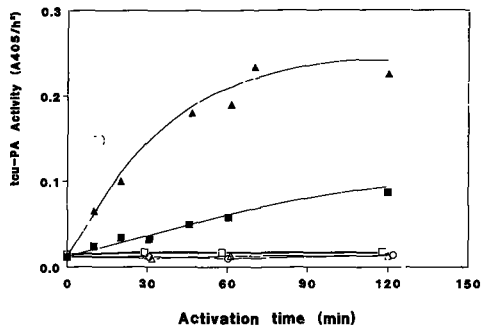

Fig 1 Time curves of the activation of scu-PA by plasmin scu-PA, immuno-immobilized to the wells of a microtitre plate, was activated with plasmin for the times indicated Subsequently, the plasminogen activator activity of the tcu-PA formed was measured as described in Materials and Methods Plasmin 0 25 nM (▲), 0.025 nM (■), buffer alone (□), plasmin (0 25 nM) in the presence of 650 KIU/ml aprotinin (○) or excess antibodies raised against plasminogen (△)

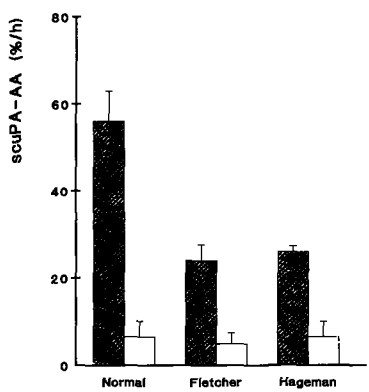

Fig. 2 scuPA-AA of DEFs of normal plasma and of plasmas deficient in prekallikrein (Fletcher trait) or factor XII (Hageman trait) After u-PA depletion of the fractions as described in Materials and Methods, scuPA-AA was measured without (hatched bars) and with 650 KIU/ml aprotinin present during activation (open bars) Error bars show standard deviations (n = 4)

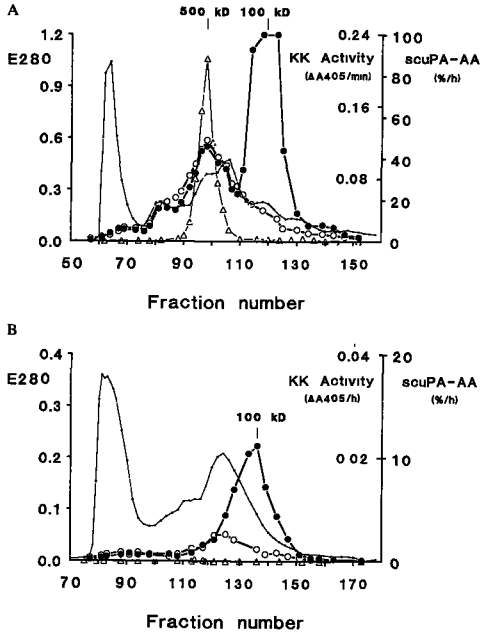

Fig 3 ACA 34 gel chromatography of DXS-euglobulin subfractions of normal plasma (A) and of plasma deficient in factor XII and subsequently immuno-depleted of prekallikrein (B) (see Materials and Methods). E280, – · – –; scuPA-AA, ●——●; scuPA-AA with excess anti-plasminogen present during activation, ○——○, kallikrein (KK) activity, △——△

Analysis of scuPA Activators in the DEF

The effect of contact activation on the scuPA-AA of plasma is shown in Fig 2 (hatched bars). The scuPA-AA present in a DEF of Fletcher and Hageman trait plasma is reduced to about 50% of that present in a DEF of normal plasma. The figure also shows that in all cases the scuPA-AA is almost completely blocked by aprotinin (Fig. 2, open bars).

The nature of the scu-PA activators present in the DEF was further investigated A DXS-euglobulin subfraction of normal plasma, obtained as described in Materials and Methods, was subjected to ACA 34 gel chromatography to separate the activators. Fractions were tested for scuPA-AA Fig. 3A (closed circles) shows that, apart from some minor peaks around M_r 800,000 and 250,000 (not further identified), two major peaks of scuPA-AA can be distinguished, one eluting with an apparent M_r of 500,000 and the other around 100,000 The former peak coincides with the activity of kallikrein in complex with kininogen (25) (open triangles). The latter peak of activity can be inhibited by antibodies raised against human plasminogen (open circles) and coincided with the activity of plasmin on S-2251 (not shown). The activity of both peaks could be abolished by aprotinin (not shown).

Results obtained upon chromatography of a DXS-euglobulin subfraction of plasma deficient in factor XII and depleted of prekallikrein are shown in Fig 3B. The 500 kDa peak of scuPA-AA has disappeared (as has the activity of kallikrein, open triangles) and only the peak around M_r 100,000 is detected. As with normal plasma this activity coincided with the peak of plasmin and could be inhibited by antibodies raised against plasminogen (Fig. 3B, open vs closed circles).

Table 1 Comparison of the scuPA-AA on a molar basis (scuPA-AA/nM activator)

Activator	Concentration tested (nM)	scuPA-AA (% min^{-1})	scuPA-AA/nM (% min^{-1} nM^{-1})
500 kDa peak from ACA 34[1]	214 21	1 7 0 3	0.008 0 014
Kallikrein subunit[2]	21	0 3	0.014
100 kDa peak from ACA 34[3]	0 35 0 05	1 5 0 2	4.3 4.0
Plasmin[2]	0 25 0 025	2.2 0 3	8 8 12.0

The kallikrein und plasmin concentrations were measured as described in Materials and Methods

[1] Fraction 98 from Fig 3A, kallikrein in complex with kininogen.

[2] Commercially obtained, see Materials and Methods.

[3] Fraction 121 from Fig. 3A, plasmin

Relative Potency of scu-PA-Activators

In order to assess the relative potency of the scu-PA activators described above, the activities of the peaks from ACA 34 and of purified preparations of kallikrein and plasmin were compared on a molar basis. Table 1 shows that at an equimolar concentration of 21 nM the purified kallikrein subunit is as effective in activating scu-PA as is the 500 kDa peak from ACA 34 containing the kallikrein-kininogen complex (0.014% min^{-1} nM^{-1}). Purified plasmin seems to be 2–3 times more effective than the 100 kDa peak from ACA 34 (8.8–12.0 vs 4 0–4.3% min^{-1} nM^{-1}), but this is trivial since the peak fraction also contained contaminating enzymes which were reactive in the plasmin assay with S-2251 and therefore resulted in an over-estimation of the plasmin concentration in Table 1. Plasmin appears to be almost 1,000 times more effective than kallikrein (12.0 vs 0.014% min^{-1} nM^{-1}) on a molar basis.

Discussion

Previous reports from our Institute (18–20) have shown that in DEFs of plasma deficient in the contact factors factor XII and prekallikrein, as much active plasma urokinase is found as in DEFs of normal plasma and it was concluded that the contact activation process is not required for the activation of the urokinase-type plasminogen activator in plasma. The recent finding of Hauert et al. (16) that kallikrein is the dominant activator of scu-PA in a DEF of plasma, however, seems to conflict with the earlier reports. Therefore, we decided to re-investigate this matter and set up an assay system that specifically measures scu-PA activators. We applied a technique originally described by Exner et al. (29) for the detection of protein C activators In this technique the substrate to be activated (here scu-PA) is first immuno-immobilized to the wells of a microtitre plate and then activated for a certain period of time with the activator. Subsequently, the amount of the immobilized active enzyme (here tcu-PA) is detected (here with plasminogen and S-2251). The sensitivity of this assay system allowed us to make use of physiological concentrations of scu-PA (50 pM, refs. 7, 26, 30–32) Results obtained upon activation with the familiar

activator of scu-PA, plasmin, showed a time- and concentration-dependent activation of the immobilized scu-PA (Fig. 1). Within an activation period of one hour we were able to detect the effect of as low as 25 pM plasmin.

When we applied this assay system to assess the effect of contact activation on the development of scuPA-AA in DEFs (Fig. 2), we found that with factor XII- and prekallikrein-deficient plasma a portion of activity of about 50% was still present that obviously does not depend on contact activation. This is a sufficiently high activity to explain our previous findings that fully activated tcu-PA can be found in the DEFs of the respective deficient plasmas (18–20). In order to determine whether activated factor XII, kallikrein or both would contribute to the other 50% of scuPA-AA, we also measured in the presence of aprotinin which inhibits kallikrein, but not factor XII_a. Since the scuPA-AA could almost completely be inhibited (Fig. 2, open bars), we conclude that factor XII_a does not substantially contribute, confirming the findings of Ichinose (12) and Hauert (16).

Subsequently, we separated the scu-PA activators in the DEF by ACA 34 gel chromatography of a subfraction (Fig. 3) and identified them as plasmin and kallikrein (in complex with kininogen). Therefore, we conclude that plasmin and the contact-activation factor kallikrein each contribute for about 50% to the scuPA-AA in the DEF.

Finally, we compared the activity of the different activators on a molar basis (Table 1). It became apparent that plasmin is almost 1,000 times more effective in activating scu-PA than is the M_r 500,000 kallikrein-kininogen complex. This was not due to the complexation of kallikrein with kininogen, since both the kallikrein subunit and the 500 kDa kallikrein-kininogen complex scored, at equimolar concentration (Table 1), an equal scuPA-AA of 0.014% min^{-1} nM^{-1}. A much smaller ratio (1.5; refs. 10, 12, 16) for the ability of plasmin over kallikrein to activate scu-PA has been reported before, but in that instance the activities were measured with scu-PA in solution (10, 12, 16) and at a much higher concentration (600 nM, ref. 12). Apparently, the conditions of our assay system, where the substrate scu-PA is tested at physiological concentration (50 pM) and in an immobilized form, must be in favour of plasmin. Since, in the freshly isolated DEF, the concentration of kallikrein is much higher (up to 500 nM) than that of plasmin (<1 nM), it can now be understood that the investigators using the non-immobilized scu-PA (13–16) mainly measured the effects of kallikrein, while in this paper, with the immobilized scu-PA, the contributions of kallikrein and plasmin to the scuPA-AA are equal.

In recent years it has been shown (see e. g. ref. 33) that many types of cells, including the endothelial cells of the vessel wall (34, 35), possess receptors with a high affinity for scu-PA on their membranes and that under physiological conditions scu-PA may be activated while bound (36, 37), thus giving rise to local fibrinolysis. The conditions in our assay system, more or less, mimic the activation of surface-bound scu-PA and the results indicate that then plasmin will be the more effective activator. In vivo, after local initiation of the contact system by a specific trigger (e. g. a negatively charged surface on the vessel wall), nearby bound scu-PA might be activated in a cascade-like manner via kallikrein, but, next to that, the formed tcu-PA will immediately generate plasmin, which in effect will outrival kallikrein almost 1,000-fold. Therefore, we consider that plasmin is the primary activator of scu-PA and the role of the contact system is of secondary importance.

Acknowledgements

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CHAPTER VI

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

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

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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.

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

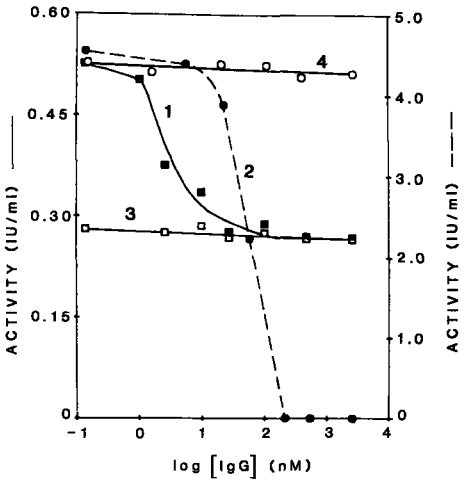

Figure 1. Inhibition of plasminogen activator activity by increasing amounts of AUK and anti-t-PA antibodies. Plasminogen activator activity was measured by the fibrin plate method (as described in Materials and Methods). Five microliters of different dilutions of an AUK IgG stock (concentration 16.5 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

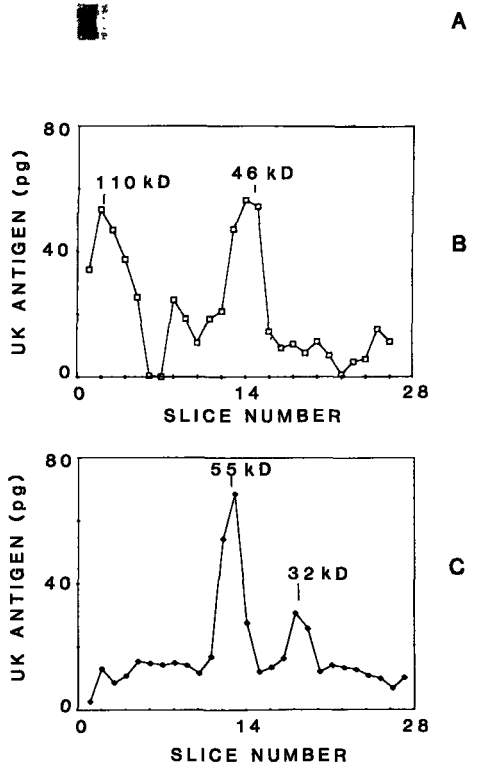

Figure 2. SDS-PAGE of urinary UK and of plasma immunodepleted in UK. Fifty microliters of a DEF of pooled plasma immunodepleted in UK (A,B) and 3 ng urinary UK (C) were subjected to SDS-PAGE (see Materials and Methods). Hereafter the protein was stained in (A), and in (B,C) the gels were sliced and extracted 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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CHAPTER VII

THE CONTACT-SYSTEM DEPENDENT PLASMINOGEN ACTIVATOR FROM HUMAN PLASMA: IDENTIFICATION AND CHARACTERIZATION

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The Contact-System Dependent Plasminogen Activator from Human Plasma: Identification and Characterization

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Summary

Apart from tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), a third PA appears to occur in human plasma. Its activity is initiated when appropriate triggers of the contact system are added, and the activation depends on the presence of factor XII and prekallikrein in plasma. The activity of this, so-called, contact-system dependent PA accounts for 30% of the PA activity in the dextran sulphate euglobulin fraction of plasma and was shown not to be an intrinsic property of one of the contact-system components, nor could it be inhibited by inhibitory antibodies against t-PA or u-PA. We have succeeded in identifying this third PA in dextran sulphate euglobulin fractions of human plasma. Its smallest unit (SDS-PAGE) is an inactive 110 kDa single-chain polypeptide which upon activation of the contact system is converted to a cleaved, disulphide-bridged molecule with PA activity. The native form, presumably, is an oligomer, since the apparent M_r on gelchromatography is 600,000. The IEP is 4.8, much lower than that of t-PA and u-PA. Although the active 110 kDa polypeptide cannot be inhibited by anti-u-PA, it yet comprises a 37 kDa piece with some u-PA related antigenic determinants. However, these determinants are in a latent or cryptic form, only detectable after denaturation by SDS. The 110 kDa polypeptide is evidently not a dimer of 55 kDa u-PA or a complex of u-PA with an inhibitor. It is probably a PA derived from a gene quite distinct from that of t-PA or u-PA, but sharing some homology with u-PA. The physiological role of this contact-system dependent PA remains to be established.

Introduction

In the circulation fibrin has a temporary function and has to be lysed in due time. Fibrinolysis is catalyzed by the enzyme plasmin, which is formed from the inactive zymogen plasminogen through limited proteolysis by plasminogen activators (1, 2). In the dextran sulphate euglobulin fraction of plasma two portions of plasminogen activator activity can be distinguished: i. a portion already present in the plasma taken from individuals at rest and ii. an additional portion, found after stimuli such as venous occlusion or exercise (3). The latter portion is called the extrinsic activity and comes from tissue-type plasminogen activator (t-PA) (4). The former portion is called the intrinsic activity and consists of activators which apparently circulate in the blood as proenzymes (3). 50% of this activity comes from plasma urokinase (UK) (5, 6), a urokinase-type plasminogen activator (u-PA) discovered in the early 80's (7, 8).

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The remaining 50% of intrinsic activity can neither be inhibited by antibodies against t-PA, nor by anti-u-PA (9, 10). This type of activity comes about by activation of the contact system [by the addition of dextran sulphate (DXS)] and is absent in plasmas deficient in factor XII or prekallikrein (11). Activated factor XII and kallikrein are capable of activating plasminogen (10–13), although their specific activity is at least four orders of magnitude lower than that of u-PA (10, 14–16). When an inventory of their contribution was made, they accounted in total for 15% of the intrinsic activity (10, 17). Apart from this contribution factor XII and prekallikrein also seem to be involved in the activation of a postulated, but as yet unidentified, plasminogen proactivator (11). The resulting, so called, contact-system dependent plasminogen activator would contribute for the 35% of intrinsic activity that remains to be explained (10, 11, 17).

In a previous paper (9) we described that dextran sulphate euglobulin fractions, devoid of t-PA and u-PA, but still containing the contact-system dependent plasminogen activator activity, contain protein that after denaturation by SDS can react with anti-u-PA. This protein cannot in native form be removed by immunodepletion over anti-u-PA Sepharose columns. Apparently it contains u-PA related antigenic determinants in a latent or cryptic form which upon unfolding become accessible to, and immunoreactive with, the anti-u-PA. On SDS-polyacrylamide gels the unmasked immunoreactivity (Western blot analysis with anti-u-PA, ref. 18) comigrates with plasminogen activator activity (fibrin zymography) at 110 kDa.

In this study we further investigated the properties of this 110 kDa plasminogen activator. We found that it is a proactivator whose activation is dependent on the activation of the contact system and that the activity of the active form may account for the portion of intrinsic activity ascribed to the previously postulated plasminogen activator. Part of this work has been presented before (19).

Materials and Methods

The following materials were obtained from the indicated sources:

Dextran sulphate (M_r 500,000), protein-A Sepharose-4B and low molecular weight markers for electrophoresis from Pharmacia, Uppsala, Sweden. Benzamidine hydrochloride hydrate from Aldrich-Europe, Beerse, Belgium. Aprotinin (Trasylol) from Bayer, Leverkusen, FRG. LKB Ampholine electrofocusing column, LKB 1809 Ampholine carrier ampholytes pH 4–6, ultrogel ACA 34 from L.K.B., Bromma, Sweden. Ammonium sulphate p.a. and molecular weight markers (MS-11) for gel chromatography from Serva, Heidelberg, FRG. S-2251 from KabiVitrum, Stockholm, Sweden. Chromozyme pK or Bz-Pro-Phe-Arg-pNA AcOH (PPAN) from Pentapharm A.G., Basle, Switzerland. Urinary urokinase from Leo Pharmaceutical Industries, Ballerup, Denmark and from Choay, Paris, France. Human plasminogen, CNBr-fragments of fibrinogen, rabbit anti-plasminogen activator inhibitor (PAI)-1 IgGs, rabbit anti-plasminogen antiserum, and rabbit anti-tissue-type plasminogen activator IgGs were kindly provided by, respectively,

Drs D W Traas, W Nieuwenhuizen, E D Sprengers, D C Rijken and J. H Verheijen from our Institute.

Plasminogen-free bovine fibrinogen was obtained from Poviet, Organon Teknika, Oss, The Netherlands

Plasminogen-rich bovine fibrinogen was prepared according to Brakman (20). Rabbit and goat anti-u-PA IgGs isolated from antisera raised against an urinary UK preparation were obtained and affinopurified as described previously (6).

Antisera raised against prekallikrein, factor XII and kininogen were obtained from Nordic, Tilburg, The Netherlands.

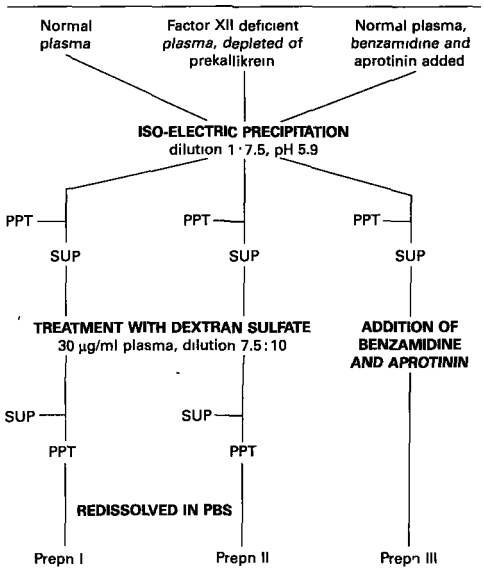
Platelet-poor citrated plasma was prepared as described by Kluft et al (21). Dextran sulphate euglobulin fraction of plasma (DEF) was prepared according to Kluft et al. (22).

Plasma deficient in factor XII, was kindly provided by Dr E. J P Brommer from our Institute and contained no detectable factor XII (23).

Plasmas artificially depleted of, respectively, prekallikrein, u-PA or t-PA were prepared by immunoadsorption with anti-prekallikrein IgGs, anti-u-PA IgGs or anti-tPA IgGs coupled to Sepharose-4B, similarly as described before for depletion of UK (8) After depletion, in the respective plasmas the concentration of prekallikrein, u-PA or t-PA was below the lower limit of sensitivity of the assays used (24–26) This implies that in all cases depletion occurred for at least 97%

Fractionation of Plasmas

Preparations I, II and III were prepared from, respectively, normal plasma, factor XII deficient plasma depleted of prekallikrein, and normal plasma as indicated in Scheme I Iso-electric precipitation and treatment with dextran sulphate were carried out as described by Kluft et al. (11). Subsequently Prepns I–III were further fractionated as indicated in Scheme II. Ammonium sulphate fractionation, to remove most of the fibrinogen, was carried out according to Kluft et al (11) Protein-A Sepharose gelchromatography (1 5 × 2 5 cm column), ACA 34 gelchromatography and iso-electric fractionation (LKB 8100 *Ampholine* electrofocusing column) were carried out according to the prescriptions of the manufacturers Throughout the fractionation procedure of Prepn III the protease inhibitors aprotinin (15 KIU/ml) and benzamidine (10 mM) were present



Scheme I Fractionation of plasmas

SDS-PAGE

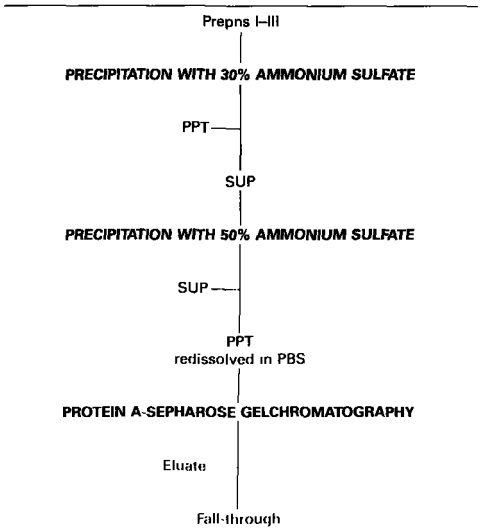
SDS-PAGE of purified Prepns I–III was carried out on slab gels according to Laemmli (27) with a 5% stacking gel and a 8 5 or 10% running gel

In some cases, prior to electrophoresis, samples were treated with 8 M urea, 4 M guanidine hydrochloride or with 0 1% SDS followed by 2 M hydroxylamine according to, respectively, Erickson et al (28) and Levin (29) To these samples (200 µl) the protease inhibitor aprotinin in a final concentration of 200 KIU/ml, had been added in advance The treated samples were dialysed against 0 05 M Tris-HCl pH 8 0 containing 0 1% (v/v) Tween 80 and 10 KIU/ml aprotinin for 18 h at 4°C Hereafter samples were mixed with an equal volume of sample buffer (27)

Reduced samples were obtained with a sample buffer that contained β-mercapto-ethanol [4% (v/v)] The mixtures were immersed for 2 min in boiling water applied to the slab gel and electrophoresed overnight at 3 mA For calculation of molecular weights, low molecular weight markers were electrophoresed in parallel lanes After electrophoresis was completed these lanes were cut off and the marker proteins were stained with Coomassie Brilliant Blue as described before (9)

Detection and quantification of the 110 kDa activator occurred as follows

Western blot analysis In some experiments the 110 kDa activator antigen was estimated semi-quantitatively by immunoblotting with anti u-PA (18) Protein was transferred to nitrocellulose sheets essentially by the method of Towbin (30) Transfer was carried out using the Bio Rad Trans Blot cell filled with Tris (25 mM) glycine (192 mM) pH 8 3, 20% (v/v) methanol for 2 h at 400 mA The blotted nitrocellulose sheets were washed with buffer [10 mM Tris HCl, pH 7 4 0 9% (m/v) NaCl 0 05% (v/v) Tween 20] with 3% bovine serum albumin Subsequently the sheets were incubated for two hours at room temperature with goat anti u PA IgGs washed with buffer, incubated with peroxidase-linked rabbit anti goat IgGs and again washed with buffer u-PA related antigen was then visualized with 4 chloronaphthol as follows the nitrocellulose sheets were



Scheme II Fractionation of preparations I–III

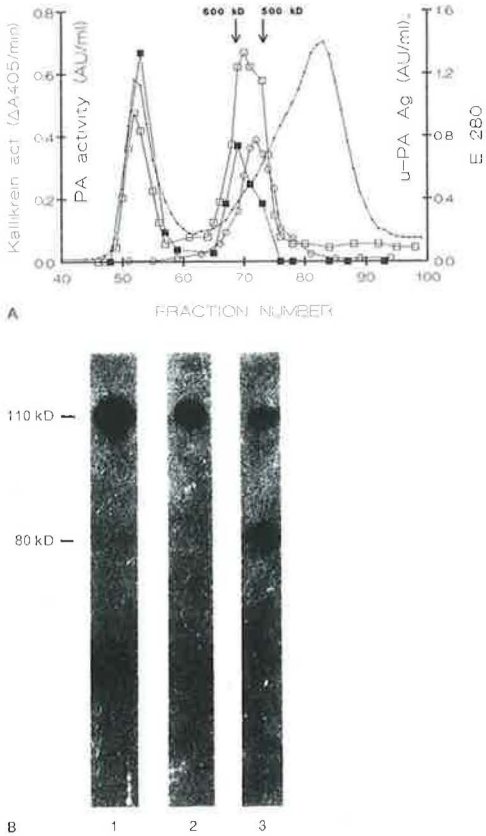


Fig. 1. Gelchromatography of Prepn I (A) and fibrin-agarose zymography of peak fractions (B). (A): Prepn I, prepared and fractionated from 100 ml plasma (Schemes I, II) was subjected to ACA 34 gelchromatography (100 × 2 cm column, fractions of 2.8 ml) in PBS. Elution profiles are shown of: E 280 (——), residual plasminogen activator activity in the presence of excess anti-u-PA and anti-t-PA antibodies (–□–), kallkrein activity (–○–), and u-PA related antigen detected by Western blot analysis at 110 kDa (–■–). (B): Zymograms of fractions 52, 68, 73 from A

incubated with 0.01% H_2O_2 in a 1:5 mixture (v/v) of a solution of 4-chloronaphthol (0.3%, m/v) and 50 mM Tris-HCl pH 7.4, 200 mM NaCl. Incubation was performed at room temperature, usually for about 15 min, until stained protein bands were visible. Then the reaction was stopped by rinsing with tap water.

The amount of u-PA-related antigen was estimated semi-quantitatively by comparing the area and intensity of the bands with area and intensity of bands obtained with increasing concentrations of 55 kDA urinary UK from Choay.

ii. Immuno assay. In the experiment of Fig. 5 lanes were cut out from the running gel and washed for 2 h at 37° C in 2.5% Triton X-100. Then the lanes were sliced (34 slices of 0.32 cm) and the slices were extracted by incubating for 48 h with 250 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% (v/v) Tween 80 and 0.02% (m/v) NaN_3, as described before (9). In these slice extracts u-PA activity was detected with a biological immuno assay (BIA, ref. 31) for u-PA (32). In this assay u-PA-related protein is immuno-immobilized to the wells of a PVC microtiterplate and subsequently the activity of the immobilized material is measured. Immuno-immobilization of the u-PA-related material in the BIA was performed in the same way and with the same anti-u-PA antibodies as described for the sample incubation step of the ELISA for u-PA (25). Plasminogen activator activity of the immobilized protein was detected with plasminogen and S2251, in essence as described by Verheijen et al. (33) for the measurement of t-PA in a soluble system. The rate of release of p-nitroanilne (pNA) was determined at 405 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Irvine, Scotland). Activity was calculated by reading the change in absorbance per square hour from calibration curves obtained with Leo UK.

iii. Zymography. In other experiments slab gels were washed for 2 h at 37° C in 2.5% Triton X-100. Subsequently, gels were transferred to plasminogen-rich fibrin-agarose underlays (34) and incubated, for 18 h, at 37° C. In parallel experiments appropriate amounts (30 µg/ml) of goat anti-u-PA IgGs (9) and rabbit anti-t-PA IgGs were incorporated in the underlays to inhibit the activity of u-PA and t-PA. To allow for discrimination between plasminogen-dependent and -independent lysis of the underlays, parallel gels were transferred to plasminogen-free fibrin-agarose underlays.

Assays

Plasminogen activator activity. Plasminogen activator activity of samples was assayed with the standardized fibrin plate method (35) with excess anti-u-PA and anti-t-PA antibodies added as described before (9).

Kallikrein and prekallikrein. Kallikrein activity was determined, in essence according to Kluft et al. (24), using the chromogenic substrate for kallikrein, PPAN. 10 µl samples were incubated with 200 µl Tris-imidazole buffer (µ = 0.15; pH 7.9) with 0.1% Carbowax and 40 µl PPAN (1 mM in distilled water). All incubations were performed at 37° C in microtiterplates. The absorbance at 405 nm was recorded with a Titertek Multiskan spectrophotometer. Activity was expressed as the change in absorbance per minute and was calculated from the linear of the curve.

Prekallikrein was assayed after conversion to kallikrein. Prekallikrein in plasma was converted by incubation of 25 µl plasma and 25 µl dextran sulphate solution (25 µg/ml) for 12 min at 0° C as described by Kluft et al. (24). Prekallikrein present in purified fractions was converted by incubation of 12.5 µl sample, 12.5 µl plasma artificially depleted in prekallikrein and 25 µl dextran sulphate solution. Fractions containing benzamidine, were dialysed in advance against 0.14 M NaCl, 0.01 M sodium phosphate pH 7.4 (PBS).

Factor XII. Factor XII was determined as described previously (23).

Results

Identification of Active Form

The plasminogen activator activity present in the dextran sulphate treated normal plasma (Prepn I, Scheme I), was further fractionated as described in Scheme II. On ACA 34 gelchromatography (Fig. 1A) the contact-system dependent plasminogen activator activity (the activity which cannot be inhibited by anti-u-PA or anti-t-PA, open squares) is found in the void volume of the column and around an apparent M_r of 500,000–600,000. The activity of the latter peak is partly due to kallikrein which peaks at 500,000 (open circles). The remaining activity at 600,000 and the activity in the void volume, together account for 60% of the activity in Fig. 1A and are due to a plasminogen activator which on SDS-polyacrylamide gels shows lysis of fibrin at 110 kDa (see zymograms, Fig. 1B). The 110 kDa plasminogen activator is immunoreactive with anti-u-PA on Western blots (see below, Fig. 4, lane 2) and the surface intensity of the bands of the blots (Fig. 1A, closed squares) parallels the

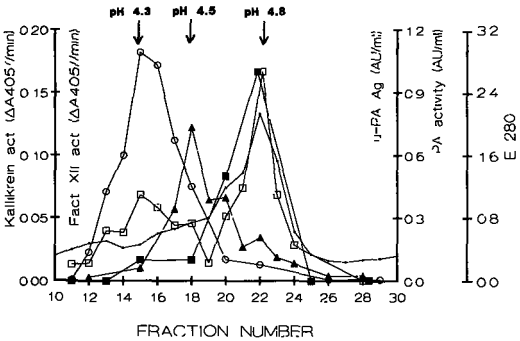


Fig 2 Isoelectric fractionation of Prepn I. Prepn I, prepared and fractionated from 50 ml plasma (Schemes I, II), was subjected to iso-electric fractionation with a LKB 8100 Ampholine electrofocusing column (110 ml column volume, carrier Ampholytes pH 4–6, 2 ml fractions collected) Elution profiles are shown of E 280 (——), residual plasminogen activator in the presence of excess anti-u-PA and anti-t-PA antibodies (–□–), kallikrein activity (–○–), factor XII activity (–▲–), and u-PA related antigen detected by Western blot analysis at 110 kDa (–■–)

activity. None of the activities corresponded with factor XIIa, which eluted around M, 90,000 (not shown)

On isoelectric fractionation (Fig. 2) two distinct peaks of plasminogen activator activity are found, one at pH 4.3, coinciding with the activity of kallikrein, and the other at pH 4 8 coinciding with the 110 kDa activator. Factor XIIa (closed triangles), which focuses at pH 4 5, does not substantially contribute to the plasminogen activator activity.

Identification of Inactive Zymogen

Further fractionation was also performed with Prepn II, where factor XII and prekallikrein are missing, and with Prepn III, where the dextran sulphate treatment was omitted On ACA 34 gelchromatography (Figs. 3 A and B) both preparations appear to have only little contact-system dependent plasminogen activator activity (open squares). Immunoreactivity with anti-u-PA on Western blots is again found at 110 kDa in fractions eluting around an apparent M, of 600,000. Contrary to the results in Fig. 1, no such immunoreactivity is present in the void volume of Figs. 3 A and B, but this is due to the 1 M NaCl in the elution buffer, which also abolishes the peak in the void volume of Fig 1 (not shown). Kallikrein is again found around M, 500,000 (Fig. 3 B), but now in its activatable zymogen form.

Activation by the Contact System

In Fig. 4 A the Western blot analysis of the purified Prepns I–III, before and after reduction, demonstrates the effect of contact activation on the primary structure of the 110 kDa immunoreactive polypeptide With Prepns II and III, where no contact activation could occur, a single-chain polypeptide is found: the blots are immunoreactive with anti-u-PA at 110 kDa, both before and after reduction (lanes 3, 4 vs. 7, 8) With Prepn I, however, where contact activation did occur, a cleaved polypeptide is found, the immunoreactivity shifts from 110 kDa before reduction to 37 kDa after reduction (cf lanes 2 and 6) The zymograms in Fig 4 B show that only the cleaved 110 kDa polypeptide of Prepn I (lanes b and f) has plasminogen activator activity.

Reactivity with Anti-u-PA

The plasminogen activator activity of the cleaved 110 kDa polypeptide cannot be inhibited by anti-u-PA or anti-t-PA, either on fibrin plates before SDS-PAGE (open squares in Fig 1 A), or on fibrin underlays after SDS-PAGE (lanes b and f in Fig. 4 B). Yet in Fig. 4 A it is seen that after SDS-PAGE the immobilized polypeptide on the Western blots binds anti-u-PA. Likewise it can be demonstrated, on microtiterplates, that immobilized anti-u-PA binds the soluble polypeptide after SDS-PAGE, and that the formed complex of immobilized anti-u-PA and polypeptide retains plasminogen activator activity. In Fig. 5 such an experiment was carried out on the gel slice extracts obtained after SDS-PAGE of a dextran sulphate euglobulin fraction A substantial peak of activity is seen in the gel slices around 110 kDa, whereas

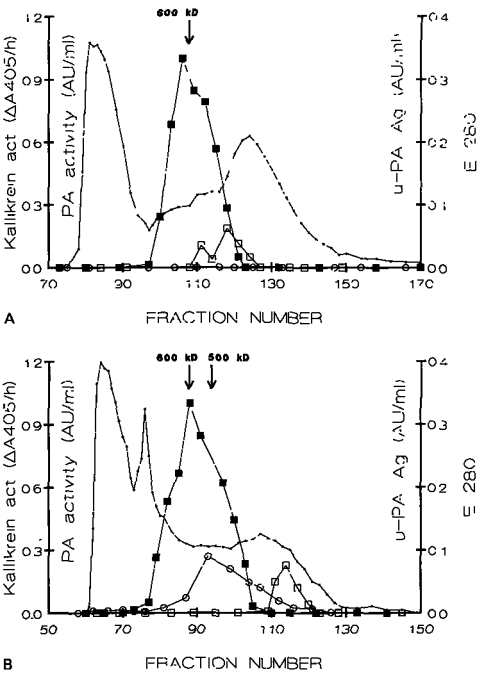


Fig 3 Gelchromatography of Prepns II (A) and III (B) (A) Prepn II, prepared and fractionated from 18 ml plasma (Schemes I, II), was subjected to ACA 34 gelchromatography [90 × 1 2 cm column, phosphate (10 mM) buffered 1 M NaCl, pH 7 4, fractions of 0 87 ml] (B) Prepn III, purified and fractionated from 75 ml plasma (Schemes I, II), was subjected to ACA 34 gelchromatography [90 × 2 cm column, in phosphate (10 mM) buffered 1 M NaCl, pH 7 4 with 10 mM benzamidine, fractions of 2 ml] Elution profiles are shown of E 280 (——), residual plasminogen activator activity in the presence of excess anti-u-PA and anti-t-PA antibodies (–□–), prekallikrein (– ○ –), and u-PA related antigen detected by Western blot analysis at 110 kDa (–■–)

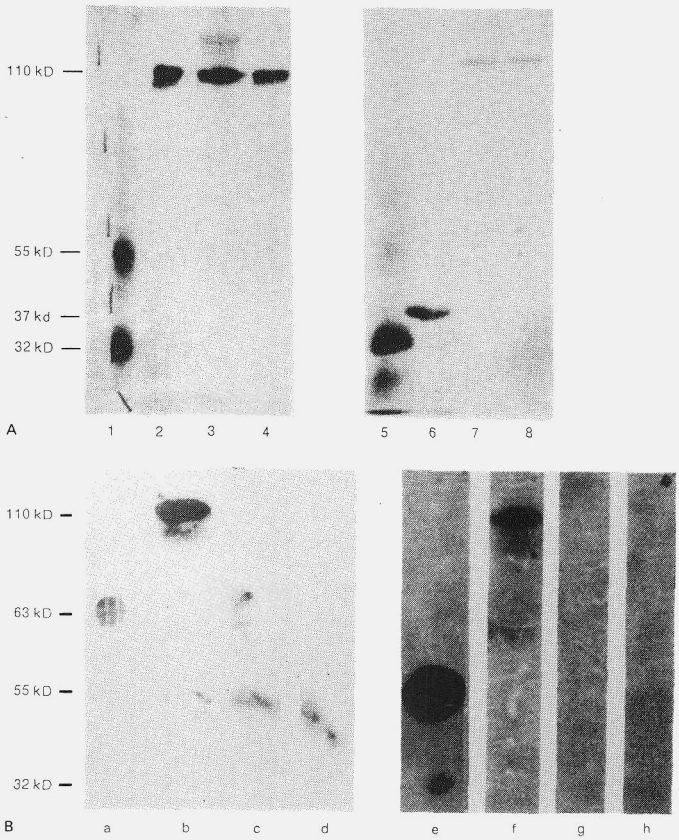


Fig. 4 Western blot analysis (A), before and after reduction, and zymography (B) *of purified Prepns I–III: effects of contact* activation. (A) Western blot analysis with anti-u-PA (10% running gel) of 25 µl samples of purified Prepns I (lanes 2, 6), II (lanes 3, 7) and III (lanes 4, 8). Lanes 1, 5 urinary UK (1 µg). In lanes 5–8 samples were treated with β-mercaptoethanol before application, as described in Materials and Methods. (B) Zymography (8.5% running gel); applied were: lanes a, e: mixture of urinary UK *(10 mIU) and t-PA (15 mIU)*: *lanes* b, f: 10 µl of purified Prepn I; lanes c, g: 25 µl of purified Prepn II; lanes d, h: 10 µl of purified Prepn III. In lanes a–d, anti-u-PA and in lanes e–h, anti-t-PA was present in the fibrin-underlay. Purified Prepns I–III are the peak fractions, numbers 68, 107 and 89, of Figs. 1A, 3A and 3B, respectively

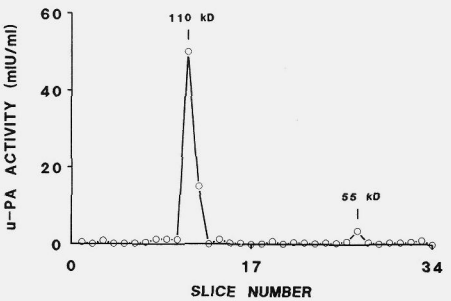


Fig. 5 u-PA related activity in slice extracts after SDS-PAGE of dextran sulphate euglobulin fraction of plasma. 25 µl of a 400% DEF of pooled plasma, immunodepleted in t-PA, were subjected to SDS-PAGE (10% running gel). Hereafter the gel was sliced and extracted with buffer; *subsequently the urokinase activity* was determined in the extracts with the immunoassay (BIA) as described in Materials and Methods

at 55 kDa plasma UK, which before immuno-immobilization accounted for 50% of the activity of this euglobulin fraction, is almost completely inhibited.

The ability of anti-u-PA to bind the 110 kDa polypeptide is an intrinsic property of polyclonal anti-u-PA preparations (three preparations raised in rabbit and goat, affinopurified against 55 kDa u-PA, were tested). Thusfar the binding phenomenon is restricted to anti-u-PA; with anti-t-PA and with antibodies against *other proteins such as plasminogen, factor XII, prekallikrein,* kininogen, PAI-1 and PAI-3 no positive staining at 110 kDa was observed. The binding concerns a 37 kDa piece of the 110 kDa polypeptide (cf. lanes 2 and 6 in Fig. 4A), not an integral 55 kDa UK molecule. Pretreatment with urea or guanidine. HCl, which *may better dissociate non-covalent protein complexes than does* SDS, had essentially the same effect as that in Fig. 4A with SDS alone (shown for guanidine. HCl in Fig. 6, lanes 2 and 3). Also pretreatment with 2 M hydroxylamine, known to dissociate covalent esterolytic bonds between e.g. serine proteases and their *inhibitors* (29), *had no further effect on the primary structure of* the 110 kDa polypeptide (see Fig. 6, lanes 4 and 5).

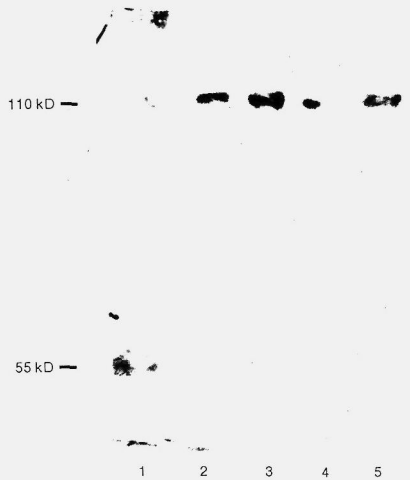


Fig. 6 SDS-PAGE of the purified Prepns I and III: effect of treatment with guanidine hydrochloride (lanes 2, 3) and hydroxylamine (lanes 4, 5). After SDS-PAGE (8% running gel; 25 µl samples) u-PA related antigen was detected by Western blot analysis. Lane 1: urinary UK (1.5 µg); lanes 2, 4: Prepn I; lanes 3, 5: Prepn III. Before application samples were treated with hydroxylamine and guanidine hydrochloride as described in Materials and Methods. Purified Prepns I, III are the peak fractions, numbers 68 and 89, of Figs. 1 A and 3 B, respectively

Discussion

Identification

During our attempts to identify the plasminogen activators in the dextran sulphate euglobulin fraction of plasma we observed that on Western blots, apart from UK, there is yet more protein in the fraction which can react with anti-u-PA (18). Since this protein, in native form, could not be removed by immunodepletion over anti-u-PA Sepharose columns, it apparently contained u-PA-related antigenic determinants in a latent or cryptic form which upon unfolding by SDS-PAGE became accessible to, and immunoreactive with, anti-u-PA (9). On SDS-polyacrylamide gels the unmasked immunoreactivity (Western blots) comigrated with plasminogen activator activity (fibrin zymography) at 110 kDa. In this report it was demonstrated (Fig. 5) that the protein in the gel slice extracts at 110 kDa could be singled out with immobilized anti-u-PA and then retained activity. This implies that immunoreactivity and plasminogen activator activity are properties of one and the same *110 kDa polypeptide*. It was consistently found that on further purification of the dextran sulphate euglobulin fraction with gelchromatography or isoelectric fractionation (Prepn I in Figs. 1 and 2) the immunoreactivity remained inseparable from the plasminogen activator activity at 110 kDa.

The plasminogen activator activity of the 110 kDa polypeptide only came to expression in Prepn I, not in Prepns II and III, where activation of the contact system was prevented (Fig. 4 B). *This means that the* 110 kDa polypeptide is indeed a contact-system dependent plasminogen activator. Its activity was separated in Figs. 1 and 2 from the activity of the other plasminogen activators known to be present in the dextran sulphate euglobulin fraction, notably from plasma UK (not shown) and from kallikrein and factor XIIa. The activity of the 110 kDa polypeptide accounted for 60% of the contact-system dependent activity in Figs. 1 and 2, which is 30% of the total activity, plasma UK included. This implies that if the recovery of the respective activators on purification is about the same, the activity of the *110 kD* polypeptide does indeed account for the 35% of plasminogen activator activity in the dextran sulphate euglobulin fraction that had yet to be explained.

The existence of a contact-system dependent fibrinolytic agent *in human plasma, distinct from factor XIIa and kallikrein*, has been described previously (36). Later Kaplan and Austen (37) described an agent with similar properties, but upon reinvestigation it was concluded that it concerned kallikrein yet (38). *Thereafter Kluft et al. (11) put forward evidence for the existence* of a factor XII- and kallikrein dependent plasminogen proactivator, *distinct from factor* XII and prekallikrein. We consider that the (pro)activator identified and characterized in this paper, *is basically the same activator as that in the preparations* described by Kluft. Recently Hauert et al. (39) made a thorough analysis of a dextran-sulphate activated euglobulin fraction on SDS-PAGE but were unable to identify an active plasminogen *activator distinct from t-PA, u-PA, factor XIIa or kallikrein*. Their failure to detect such an activator probably relates to the fact that the activator occurs in plasma as a zymogen and that at 0° C an activation period of 30 min is inadequate. Furthermore, after the SDS-PAGE the zymogen on the gels (110 kDa) had probably been sufficiently separated from factor XIIa and kallikrein to prevent its activation.

Characterization

The primary structure of the 110 kDa plasminogen activator changed upon contact activation. In the purified Prepns II and III, where contact activation had been prevented, the activator was a single polypeptide chain, as demonstrated with Western blot analysis (Fig. 4 A) and protein staining (not shown) of the gels. In the purified Prepn I, where contact activation had occurred, the activator was a cleaved, disulphide-bridged polypeptide chain which comprised a piece of 37 kDa that was immunoreactive with anti-u-PA. Apart from this piece of 37 kDa, protein staining after reduction showed a piece of about 110 kDa which apparently was not immunoreactive. Most likely these two pieces constitute the total 110 kDa plasminogen activator, which implies that its apparent M_r of 110,000 on SDS-PAGE before reduction is an underestimation, probably because of improper unfolding.

A change of primary structure upon activation is often met with serine proteases; these enzymes usually occur in the body as zymogens or so-called proactivators and disclose their active centre after cleavage of the polypeptide chain at a specific site. The 110 kDa polypeptide in this paper apparently underwent a similar structural change and most likely the purified Prepns II and III contain the inactive proactivator, while Prepn I contains the active plasminogen activator. Some evidence for a serine protease was also obtained; the purified Prepn I bound to benzamidine-Sepharose (unpublished) and similar preparations were shown earlier (18) to incorporate radiolabelled DFP.

The apparent molecular weight on gelchromatography was 600,000, both with the proactivator (Fig. 3) and the activator (Fig. 1). After SDS-PAGE the plasminogen activator activity, the immunoreactivity and most of the protein were found at the much lower apparent M_r of 110,000 (Fig. 4), suggesting that the parent molecule is an oligomer. The homogeneity of *the 110 kDa band* on SDS-PAGE, however, may be deceptive in the sense that distinct polypeptides of 110 kDa might be present.

The isoelectric point of the 600,000 plasminogen activator (Fig. 2) was 4.8, far away from the IEP of t-PA and u-PA, which is around 8.5 (40). This low IEP may be an intrinsic property of the 110 kDa polypeptide with plasminogen activator activity or may be due to its complexation with another polypeptide with low IEP. Compare for instance kallikrein, which has an IEP of 8.7 (41), and is found in Fig. 2 at an IEP of 4.3, because of complexation with kininogen (42).

A 37 kDa piece of the 110 kDa plasminogen activator was immunoreactive with anti-u-PA. This property appeared to be a unique feature of anti-u-PA in the sense that antisera raised against a series of other, more or less related, proteins did not show reactivity. As discussed earlier (9), three possibilities for the nature of the relationship between the 110 kDa plasminogen activator and u-PA may be considered: i. the 110 kDa activator is a precursor of the 55 kDa plasma UK, ii. the 110 kDa activator is 55 kDa plasma UK, covalently bound to an inhibitor, iii. the 110 kDa activator and u-PA cannot be interconverted and are distinct transcription products. The first possibility can now be ruled out since the mRNA derived from the u-PA gene has been identified and is too small (2500 nucleotides, refs. 43, 44) to account for a precursor of M_r 110,000. A tight, non-covalently bound, complex of 55 kDa plasma UK with itself or with another 55 kDa polypeptide is unlikely as treatment with urea or guanidine HCl had no effect (Fig. 6, lanes 2 and 3). The idea of a covalent complex with an inhibitor (ii) would appear to be attractive, since inhibitors may obscure antigenic determinants of the target protein. Moreover, complexes between plasminogen activators and inhibitors are able to lyse fibrin after SDS-PAGE and some of these have a M_r of about 100,000 (29, 34, 45). The 110 kDa plasminogen activator in this paper, however, was also active before SDS-PAGE and, contrary to the described activator-inhibitor complexes, could not be inhibited by anti-u-PA or anti-t-PA. Furthermore, the 110 kDa proactivator remained inactive, even after SDS-PAGE, and hydroxylamine had no effect (Fig. 6, lanes 4 and 5).

So we are left with the third possibility. One option is that, due to differential splicing, two distinct mRNAs, one coding for the 110 kDa plasminogen activator and the other for 55 kDa UK, might be derived from the u-PA gene. However, considering the size of the u-PA gene (see ref. 46), a splicing product encoding a 110 kDa polypeptide is not very likely. Another option is that the 110 kDa plasminogen activator and u-PA are the products of different genes, but with the 37 kDa piece, or part of it, as an homologous unit. The homology should be rather strong as e.g. antibodies raised against t-PA, a molecule which is closely related to u-PA (47), are not immunoreactive with the 37 kDa piece or u-PA. Examples of proteins that can be detected by "cross-reacting" antibodies, are plasminogen and lipoprotein (a), which comprise homologous kringle structures (48, 49).

Physiological Role

For many years it has been known that activation of the contact system of coagulation may also be involved in activation of fibrinolysis (10, 17, 50). In this paper a 110 kDa polypeptide from human plasma was identified as the contact-system dependent plasminogen proactivator and characterized. The concentration of this 110 kDa proactivator seems to be much higher than the 50 pM u-PA in plasma. The proactivator was easily made visible by immunoblotting with anti-u-PA following the successive stages of purification, while plasma u-PA was virtually undetectable by this method. On the basis of equal immunoreactivity with anti-u-PA, the plasma concentration would be about 0.2 μM, in line with estimations made from the A280 of the purified preparations. The specific activity of the 110 kDa activator, then would be very low (25 IU/mg), since its activity in the dextran sulphate euglobulin fraction at the most equals that of u-PA. It could be that the 110 kDa proactivator, upon contact activation in vitro, is improperly cleaved, as happens, for example, when scu-PA is cleaved by thrombin (51, 52), and that in vivo full activity would be obtained by cleavage at another site. The role of the contact system in activating the 110 kDa proactivator might then become doubtful, if other, yet unidentified, enzymes eventually would turn out to be more specific. An alternative explanation could be that the cleaved 110 kDa activator exerts a cofactor function in plasminogen activation, as does the bacterial protein streptokinase. In that case the specific activity would be a misconception. Whatever the real explanation might be, the 110 kDa activator contributes substantially to the plasminogen activator activity of the dextran sulphate euglobulin fraction, as u-PA and t-PA do. Further investigations are in progress.

Acknowledgements

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CHAPTER VIII

SUMMARY AND CONCLUDING REMARKS

INTRODUCTION

This thesis deals with the intrinsic system of fibrinolysis, a cascade of proteolytic proenzymes and enzymes in the circulation, involved in the dissolution of fibrin (1, 2, 3). At the start of the investigations (see Fig. 1) it was known that the lysis of fibrin is catalyzed by the enzyme plasmin, which has to be generated from the zymogen plasminogen (Plg). At the level of Plg activation two, approximately equal, portions of activity appeared to be involved (4, 5), one of which depended on the presence of the contact-activation components factor XII and prekallikrein (6). The other portion involved an enzyme which was immunochemically related to urokinase (7), a Plg activator originally recovered from urine (8). This thesis concerns in particular the plasminogen proactivators and activators which constitute the intrinsic system of fibrinolysis, and the mechanism of action by which the system is initiated and accelerated.

RESULTS

The investigations were started (Fig. 1, left-hand side) with the development of sensitive immunoassays for urokinase-type plasminogen activator (u-PA) which enabled us to assess, in blood plasma and subfractions thereof, the concentrations of total u-PA antigen and of the separate forms, i.e. the zymogen single-chain u-PA (scu-PA), the active enzyme two-chain u-PA (tcu-PA) and the irreversible complex of tcu-PA with inhibitors (u-PA.Inh). The advantage of the combination of these assays is that the conversion of scu-PA to tcu-PA and u-PA.Inh can be monitored and thereby the activation grade of the system. Chapters II and III (9-11) describe the set-up and properties of these assays: an ELISA, which measures total u-PA antigen, irrespective of the molecular form and a BIA (biological immunoassay), which measures tcu-PA and scu-PA. The lower limit of sensitivity of these assays amounts 0.1 ng/ml and the intra-assay variations are 6 and 4%, respectively. The pooled plasma of 25 healthy individuals was shown to contain 3.3 ng/ml total u-PA antigen, of which 2.1 ng/ml was scu-PA, 1.1 ng/ml u-PA.Inh and less than 0.1 ng/ml tcu-PA. In some groups of patients enhanced plasma levels were encountered, especially of u-PA.Inh and tcu-PA, amounting to 3.2 and 2.1 ng/ml, respectively (e.g.

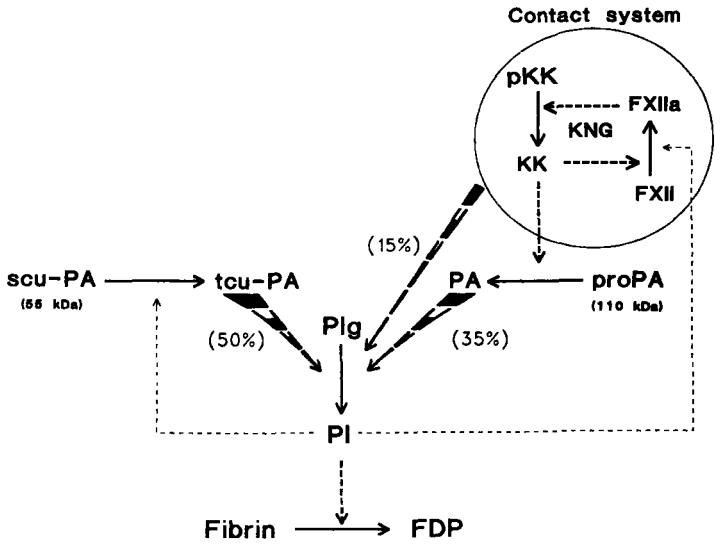

Figure 1. The intrinsic system of fibrinolysis: two co-ordinate pathways.
Abbreviations: pKK, prekallikrein: KK, kallikrein; FXII, Factor XII; FXIIa, active Factor XII; KNG, kininogen; proPA, plasminogen proactivator; PA, plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; tcu-PA, two-chain urokinase-type plasminogen activator; Plg, plasminogen; Pl, plasmin; FDP, fibrin degradation products.
Symbols: solid arrows indicate chemical conversion; broken arrows, action of proteolytic active enzyme; broken lines, feed-back action of plasmin.

liver insufficiency, see Chapter III and refs 10, 11). The occurrence of u-PA.Inh in the plasma of healthy individuals indicates that conversion of the zymogen scu-PA to active tcu-PA should have occurred and suggests that fibrinolysis in the circulation is an on-going process. The relatively increased plasma levels of tcu-PA and u-PA.Inh in some diseases show that the activation grade of the fibrinolytic system might be enhanced occasionally.

In Chapter IV (12) the occurrence of the separate forms of u-PA in blood was confirmed by isolation. About two-third of the total u-PA antigen appeared to occur as an inactive M_r 55,000 protein which could be activated by plasmin (the zymogen scu-PA), and one-third as protein of higher molecular weight which could not be activated and most likely represented tcu-PA, irreversibly bound to an inhibitor (u-PA.Inh). When isolation of u-PA was carried out from blood plasma which previously

had been activated by dextran sulphate (DXS) treatment, an active M_r 150,000 protein could be separated, which comprised active 55 kDa tcu-PA, probably in complex with another plasma component. In contrast to the other u-PA forms from plasma and the 55 kDa tcu-PA from urine, the M_r 150,000 complex showed affinity for fibrin. Since 55 kDa tcu-PA from urine also obtained affinity for fibrin when added to DXS-treated plasma, it was concluded that the fibrin affinity was brought about by the complexation. This implies that the conversion of 55 kDa scu-PA to tcu-PA in the circulation is also accompanied by the complexation and that the fibrin-bound 150 kDa complex will restrict Plg activation by u-PA to the fibrin clot.

In Chapter V (13) an analysis was made of the various possible activators of scu-PA in the blood. An assay system for scu-PA activators was set up which involves the activation of physiological concentrations of scu-PA (50 pM). It was found that plasmin and the contact-activation factor kallikrein are capable of activating scu-PA, but that on a molar basis plasmin was almost thousand times more effective. Therefore it was concluded that in the circulation plasmin would be the primary activator of scu-PA (Fig. 1, left-hand side). Moreover it was demonstrated that scu-PA activation could proceed in the plasma of patients deficient in factors of the contact system.

In Chapters VI and VII (14, 15) attention was paid to the identification and characterization of the contact-system dependent Plg proactivator and activator (Fig. 1, right-hand side). On the basis of activity measurements it had been concluded before (16) that about 50% of the Plg activator activity of the intrinsic system depended on contact activation, but that only 15% could be accounted for by the direct contributions of activated factor XII and kallikrein. Therefore it had been postulated that 35% of the activity would be due to another, not yet identified, contact-system dependent Plg activator. In order to facilitate identification of this species, plasma was first immuno-depleted of the other 50% of Plg activator activity caused by u-PA (Fig. 1, left-hand side). Much to our surprise (Chapter VI, ref. 14) it was found that the depleted plasma still contained substantial amounts of u-PA related protein, but with the antigenic determinants in a latent or cryptic form, which only upon unfolding of the protein became accessible to - and immunoreactive with - anti-u-PA. On SDS-polyacrylamide gels part of the unmasked immunoreactivity (Western blot analysis with anti-u-PA) comigrated with Plg activator activity (fibrin zymography) at 110 kDa. This finding greatly facilitated the further identifi-

cation of the postulated proactivator and activator.

In Chapter VII (15) it was found that the proactivator is an inactive single-chain 110 kDa polypeptide, which upon activation of the contact system is converted to a cleaved, disulphide-bridged, 110 kDa polypeptide with Plg activator activity. The activity of the cleaved polypeptide accounted for the 35% of activity in Fig. 1. Although the activity could not be inhibited by anti-u-PA, the 110 kDa activator comprised a 37 kDa piece which in unfolded form was immunoreactive with anti-u-PA. The apparent cross-immunoreactivity between the 110 kDa Plg activator and u-PA remained unexplained.

DISCUSSION

In this thesis it was shown that the intrinsic system of fibrinolysis may indeed proceed as originally proposed. In essence (see Fig. 1) the system consists of two co-ordinate pathways of Plg activation, each of which contributes for about 50% to the total Plg activator activity. One pathway involves the 55 kDa scu-PA and the other pathway the 110 kDa, contact-system dependent proactivator. Furthermore, each of the two pathways is subject to feed-back activation by plasmin (for the activation of factor XII by plasmin, see refs 3, 17, 18). The experiments which led to the scheme in Fig. 1, were all in vitro experiments. The question is: "What is the significance of the scheme for fibrinolysis in vivo?". In the paragraphs below the opinion is offered that the intrinsic system of fibrinolysis is initiated by the extrinsic system, proceeds upon the principle of a cascade of consecutive activation steps which accomplishes an exponential acceleration of the plasmin generation, and reinforces the impact of the extrinsic system manyfold.

From a teleological point of view the process of fibrinolysis in the circulation of healthy man must proceed locally where fibrin deposits and intact (or repaired) vessel-wall tissue go together. If this condition is not fulfilled, the process must be impaired. Thus, on the one hand the efficacy of hemostatic plugs is ensured until the vessel wall has been repaired and on the other hand undue deposition of fibrin on intact vessel-wall tissue is immediately undone. The simultaneous selectivity for fibrin and intact (or repaired) tissue is attained by the action of the extrinsic activator, t-PA, which is constitutively secreted by the endothelial cells of the

intact vessel wall (19, 20) and immediately killed off by inhibitors in the blood stream when not locally bound to fibrin (21). Since the plasmin which is generated locally by the action of fibrin-bound t-PA, besides lysing fibrin also starts initiating the intrinsic system of fibrinolysis (see also below), this system by consequence also obeys the criteria of selectivity. Once initiated, the intrinsic system maintains fibrin selectivity, because of the formation of the 150 kDa tcu-PA complex described in Chapter IV. Additional measures to secure tissue selectivity have not yet been described. At this point the role of the contact system may be considered. Originally investigators came across the components of the contact system by the delayed in vitro coagulation of the blood of patients deficient in these factors (22-24). Since the contact with foreign, mostly negatively charged surfaces was a prerequisite (25), the observations led investigators to think that in the circulation the contact system would be initiated by exposure to improper surfaces, as for instance happens upon injury of the vessel wall. Nevertheless, since 1959 the components of the contact system were also shown to accelerate in vitro fibrinolysis (26). The role in in vivo coagulation has always been equivocal (3, 26, 27) and was recently dismissed (28), but the role in in vivo fibrinolysis is still under consideration (29-31). In the circulation it may be that the intact vessel-wall tissue provides the proper catalytic surface for contact activation and thus secures the tissue selectivity of the intrinsic system.

Since plasmin is the leading catalyst of the Plg-proactivator activation in Fig. 1 (directly of scu-PA and indirectly of the 110 kDa proactivator), the intrinsic system is initiated as soon as the t-PA of the extrinsic system starts successfully generating plasmin. This triggering event is indispensable, as judged by the fact that in vitro, where the secretion of t-PA by the vessel wall is missing, an in plasma immersed fibrin clot is almost permanent (32, 33). Apparently the low activity of the proactivators with respect to fibrin-bound Plg (34-36) is, at their endogenous concentrations, not sufficient to accomplish autoactivation of the intrinsic system. Under basal conditions, the intrinsic system accomplishes an acceleration of the plasmin generation, which is exponential in time, because of the feed-back activation by plasmin, and which in effect exceeds the plasmin generation by t-PA manyfold, because of the much higher potential activity of the Plg proactivators (3, 16). The acceleration would evolve along two separate lines: the scu-PA and the 110 kDa proactivator pathway. However, since the feed-back activation by plasmin is

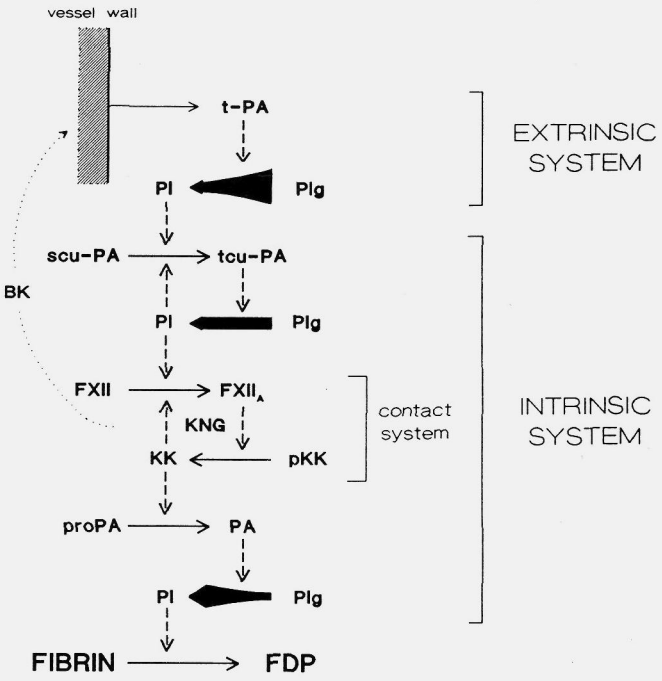

Figure 2. The fibrinolytic system: a cascade process.
Abbreviations: see legend to Fig. 1; BK, bradykinin; t-PA, tissue-type plasminogen activator.
Symbols: see legend to Fig. 1; dotted line, action of bradykinin.

mutual, the two pathways are strongly interlinked and co-operative in function. Since the concentrations of the constitutive components of the two pathways differ by several orders of magnitude (cf. scu-PA 50 pM versus factor XII 250 nM; prekallikrein 500 nM and 110 kDa proactivator 200 nM), one also could look upon the intrinsic system of fibrinolysis as if it were one cascade (see Fig. 2), starting with scu-PA on top and descending via the components of the contact system to the 110 kDa proactivator at the bottom. Such a presentation better expresses the function of the intrinsic system, which is that of an amplifier, which in no time accelerates the

plasmin generation initiated by the extrinsic system. Upon activation of the cascade the components of the contact system will also generate the hormone bradykinin (3), a split-product of kininogen, which among other functions is able to induce acute release of t-PA from the vessel wall (19, 27). Thus, additionally, local stores of t-PA will be opened up, which in potention may exceed the constitutive secretion of t-PA. It is in this way that the extrinsic and intrinsic system of fibrinolysis may co-operate and reinforce each other in the rapid and efficient removal of fibrin deposits from intact and repaired vessel-wall tissue.

Hopefully the opinion offered in this discussion may be of use to other investigators in the field and encourage them to further elucidate the mechanism of fibrinolysis.

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SAMENVATTING

Dit proefschrift handelt over het intrinsieke systeem van de fibrinolyse, een cascade van proteolytische proenzymen en enzymen in de bloedsomloop die betrokken is bij de afbraak van fibrine. Bij de start van dit onderzoek (Hoofdstuk I) was reeds bekend dat het oplossen van een fibrinestolsel wordt gekatalyseerd door het enzym plasmine, dat eerst gevormd moet worden uit het niet-actieve proenzym plasminogeen. Bij de plasminogeen activering waren twee, ongeveer even grote, porties van activiteit betrokken, waarvan één afhankelijk was van de aanwezigheid van de contactfactoren factor XII en prekallikreine. De andere portie betrof een enzym dat immunologische verwantschap vertoonde met urokinase, een plasminogeenactivator welke eerder in urine werd aangetroffen. Dit proefschrift gaat met name in op de plasminogeenproactivatoren en -activatoren die deel uit maken van het intrinsieke systeem van de fibrinolyse en de manier waarop dit systeem geïnitieerd en versneld wordt.

Het onderzoek werd gestart met de ontwikkeling van gevoelige immunologische bepalingsmethoden voor urokinase-type plasminogeenactivator (u-PA). Hiermee kon in bloedplasma en daaruit verkregen fracties de concentratie van het u-PA antigeen bepaald worden, alsmede de concentraties van de afzonderlijke moleculaire vormen, te weten het éénketenige proenzym (scu-PA), het actieve, tweeketenige enzym (tcu-PA) en het irreversibele complex van tcu-PA met remmers (u-PA.Inh). Het voordeel van de combinatie van deze bepalingsmethoden is dat de omzetting van scu-PA in tcu-PA en u-PA.Inh kan worden gevolgd en daarmee de mate van activering van het systeem. In de Hoofdstukken II en III wordt de opzet en de eigenschappen van deze methoden beschreven: een ELISA waarmee al het u-PA antigeen, onafhankelijk van de moleculaire vorm, wordt gemeten en een BIA (biologische immunoassay) waarmee tcu-PA en scu-PA apart worden bepaald. De detectielimiet van deze bepalingsmethoden bedraagt 0,1 ng/ml en de intra-assay variatie respectievelijk 6 en 4%. Het gepoolde plasma van 25 gezonde personen bleek in totaal 3,3 ng/ml u-PA antigeen te bevatten, waarvan 2,1 ng/ml scu-PA was, 1,1 ng/ml u-PA.Inh en minder dan 0,1 ng/ml tcu-PA. In sommige patiëntengroepen werden verhoogde gehaltes gevonden, speciaal van u-PA.Inh en tcu-PA, tot respectievelijk 3,2 en 2,1 ng/ml (bijv. leverinsufficiëntie, Hoofdstuk III). Het vóórkomen van u-PA.Inh in het plasma van gezonde

personen duidt er op dat omzetting van het proenzym scu-PA naar het actieve enzym tcu-PA moet hebben plaatsgevonden en suggereert dat er voortdurend fibrinolyse in de bloedsomloop plaatsvindt. De relatief hoge gehaltes aan tcu-PA en u-PA.Inh bij sommige ziektebeelden laten zien dat er soms sprake kan zijn van een verhoogde activering van het fibrinolytische systeem.

In Hoofdstuk IV werd het vóórkomen van de afzonderlijke vormen van u-PA in bloed bevestigd door deze te isoleren. Ongeveer twee-derde van het totale u-PA antigeen bleek voor te komen in de vorm van een inactief, M_r 55.000 eiwit dat geactiveerd kon worden door plasmine (het zymogeen scu-PA), en één-derde als eiwit met een hoger molecuulgewicht dat niet geactiveerd kon worden en hoogstwaarschijnlijk tcu-PA was, irreversibel gebonden aan een remmer (u-PA.Inh). Wanneer u-PA werd geïsoleerd uit bloedplasma dat eerst was geactiveerd door een behandeling met dextraansulfaat, dan werd een actief, M_r 150.000 eiwit verkregen dat bestond uit actief 55 kDa tcu-PA, waarschijnlijk in complex met een andere plasmacomponent. In tegenstelling tot de andere vormen van u-PA uit plasma en het 55 kDa tcu-PA uit urine, vertoonde het M_r 150.000 complex affiniteit voor fibrine. Aangezien 55 kDa tcu-PA uit urine ook affiniteit voor fibrine verkreeg wanneer het aan dextraansulfaat-behandeld plasma werd toegevoegd, werd er geconcludeerd dat de fibrine affiniteit door de complexering tot stand werd gebracht. Dit betekent dat ook in de bloedsomloop de omzetting van 55 kDa scu-PA in tcu-PA gepaard gaat met complexering en dat het aan fibrine gebonden 150 kDa complex de plasminogeenactivering door u-PA zal beperken tot het fibrinestolsel.

In Hoofdstuk V werd onderzocht welke de activatoren van scu-PA in het bloed zijn. Hiertoe werd een bepalingsmethode voor scu-PA-activatoren opgezet waarin gebruik gemaakt wordt van de activatie van fysiologische concentraties scu-PA (50 pM). Het bleek dat plasmine en de contactactiveringsfactor kallikreine in staat zijn scu-PA te activeren, maar dat op molaire basis plasmine bijna duizend keer effectiever is. Daarom werd geconcludeerd dat in de bloedsomloop plasmine de hoofd-activator van scu-PA is. Bovendien werd aangetoond dat scu-PA-activering kan verlopen in het plasma van patiënten die deficiënt zijn in factoren van het contactsysteem.

In de Hoofdstukken VI en VII werd aandacht besteed aan de identificatie en karakterisering van de contactsysteem-afhankelijke plasminogeenproactivator en -activator. Reeds eerder was er op basis van activiteitsmetingen geconcludeerd dat ongeveer 50% van de plasminogeenactivatoractiviteit van het intrinsieke systeem

afhankelijk was van activering van het contactsysteem, maar dat slechts 15% van deze activiteit kon worden toegeschreven aan de directe bijdragen van geactiveerd factor XII en kallikreine. Daarom was er gepostuleerd dat 35% van de activiteit veroorzaakt moest worden door een andere, nog niet geïdentificeerde, contactsysteem-afhankelijke plasminogeenactivator. Om de identificatie van deze activator te vergemakkelijken werd plasma eerst, door middel van immuno-absorptie, gedepleteerd in u-PA, dat de andere portie van 50% van de plasminogeenactivatoractiviteit uitmaakt. Tot onze verrassing (Hoofdstuk VI) werd gevonden dat het gedepleteerde plasma nog steeds een substantiële hoeveelheid aan u-PA verwant materiaal bevatte, maar met de antigene determinanten in een verborgen vorm, die alleen na ontvouwen van het eiwit toegankelijk werd voor - en immunoreactief met - anti-u-PA-antilichamen. Op SDS-polyacrylamide gels comigreerde een deel van deze immunoreactiviteit (Western blot met anti-u-PA) met plasminogeenactivatoractiviteit (fibrinezymografie) bij 110 kDa. Deze vondst vergemakkelijkte de verdere identificatie van de gepostuleerde proactivator en activator.

In Hoofdstuk VII werd gevonden dat de proactivator een inactief, éénketenig 110 kDa polypeptide is, dat bij activering van het contactsysteem omgezet wordt in een geknipt, door disulfide bruggen verbonden, 110 kDa polypeptide met plasminogeenactivatoractiviteit. De activiteit van het geknipte polypeptide was verantwoordelijk voor de 35% van de plasminogeenactivatoractiviteit die nog verklaard moest worden. Alhoewel de activiteit niet door anti-u-PA geremd kon worden, bevatte de 110 kDa activator een 37 kDa groot gedeelte dat in ontvouwen toestand immunoreactief was met anti-u-PA. De klaarblijkelijke kruisreactiviteit van de 110 kDa plasminogeenactivator en u-PA bleef onverklaard.

In Hoofdstuk VIII werden de resultaten samengevat. Er werd geconcludeerd dat het intrinsieke systeem van de fibrinolyse via twee gecoördineerde reactiepaden verloopt, die ieder voor ongeveer 50% bijdragen aan de totale plasminogeenactivatoractiviteit. Bij het ene pad is het 55 kDa scu-PA betrokken en bij het andere pad de 110 kDa, contactsysteem-afhankelijke proactivator. Beide paden zijn aan "feedback" activering door plasmine onderhevig, het eerste direct en het tweede indirect via activering van factor XII. De experimenten welke leidden tot het reactieschema waren alle in vitro experimenten. De betekenis van het reactieschema voor de fibrinolyse in vivo werd bediscussieerd. Het concept werd naar voren gebracht dat: 1. het intrinsieke systeem van de fibrinolyse op gang wordt gebracht door plasmine dat

gevormd wordt door de werking van het extrinsieke systeem (weefsel-type plasminogeen activator, t-PA, dat constitutief gesecerneerd wordt door de endotheelcellen van de vaatwand en in aanwezigheid van fibrine actief is), 2. het intrinsieke systeem van de fibrinolyse verloopt volgens het principe van een cascade van achtereenvolgende activeringsstappen, die een exponentiële versnelling van de vorming van plasmine tot stand brengt en 3. het intrinsieke systeem van de fibrinolyse dientengevolge de werking van het extrinsieke systeem vele malen versterkt. Door de gelijktijdige activering van het contactsysteem wordt tevens uit kininogeen het hormoon bradykinine vrijgemaakt, dat vervolgens de plaatselijke uitstoot van veel meer t-PA uit de endotheelcellen van de vaatwand induceert. Op deze manier werken het intrinsieke en het extrinsieke systeem van de fibrinolyse met elkaar samen en versterken zij elkaars werking zodat lokale afzettingen van fibrine op de vaatwand op een snelle en efficiënte manier verwijderd kunnen worden.

NAWOORD

Met het voltooien van dit proefschrift sluit ik een periode van onderzoek af, uitgevoerd bij de afdeling Fibrinolyse van het Gaubius Instituut TNO. Velen hebben direct of indirect bijgedragen aan de totstandkoming van dit werk. Een aantal van hen wil ik hier met name noemen:

In de eerste plaats Dr. Gerard Dooijewaard, mijn co-promotor, die op een enthousiaste en stimulerende wijze het onderzoek begeleid heeft en veel moeite heeft gestoken in het "leesbaar" maken van de manuscripten. Veel heb ik opgestoken van de discussies met hem over de diverse aspecten van het onderzoek.

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