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Radioimmunoassay of Urokinase for Quantification of Plasminogen Activators Released in Ovarian Tumour Cultures*

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Abstract—*A radioimmunoassay was developed with an urokinase antiserum and radioiodinated DFP-inactivated urokinase of* 31,000 daltons. *Assays of tissue culture medium from ovarian tumours and fetal kidneys showed the presence of urokinase-like immunoreactivity, which could be separated in three molecular forms at gel filtration. All three forms showed inhibition curves parallel to that of purified urokinase. The concentration obtained by the assay corresponded to the enzymatic activity. Distinctly parallel curves were found for tumour cultures of various ages in which the three molecular forms differed in their relative concentrations.*

INTRODUCTION

THERE is evidence that plasminogen activators are of importance for the existence of certain experimental and human neoplasms [1–10]. Such activators are released from experimental and human tumours maintained in tissue culture as determined by the appearance of fibrinolytic activity in the culture medium [5, 7, 11]. In a recent report we described that the plasminogen activator cross-reacted with an antiserum against urokinase [11]. We report here the development of a radioimmunoassay for urokinase and further immunological studies of urokinase-like plasminogen activators released in tissue culture of human fetal kidneys and human ovarian tumours.

MATERIALS AND METHODS

Urokinase was purified by affinity chromatography on *p*-amino-benzamidine-Sepharose as described in detail elsewhere [12]. 500,000 Ploug units of Urokinase Reagent (Löven, Copenhagen) was applied to the column at pH 7.0 and urokinase eluted at pH 4.0. This preparation contains three types of urokinase (approximate molecular weights 31,000, 54,000 and 100,000 daltons, respectively) which were separated by chromatography with 0.05 M Tris–HCl (pH 7.8), 0.3 M NaCl buffer on a 1.6 × 100 cm column of Sephadex G-100 Superfine, The 31,000 dalton fraction gave only one visible band when analysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It was used for immunization and as standard in the assay. For radioiodination this fraction was inactivated with diisopropyl fluorophosphate DFP [13, 14].

Urokinase antiserum was raised in rabbits with the 31,000 dalton form of urokinase as described in detail elsewhere [12]. The antiserum used in the assay was harvested after two months. In double immunodiffusion test precipitation lines of complete identity were obtained with this antiserum also against the 100,000 and 54,000 dalton forms of urokinase.

[^{125}I]-urokinase was prepared with the lactoperoxidase method according to Thorell and Johansson [15] with the following modifications. The purified urokinase (0.2 mg) dissolved in 1 ml of 0.075 M Tris–HCl (pH 7.5) was inactivated by incubation with 100 µl of 10^{-2}M DFP in propylene glycol for 3 hr [13, 14]. Non-reacted DFP was removed by gel filtration on a small column of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala). No enzymatic activity was afterwards detectable on

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plasminogen containing fibrin plates [16]. DFP-inactivated urokinase (0.02 mg) was iodinated with 37 MBq ^{125}I (IMS 30, Radiochemical Centre, Amersham, U.K.) The total reaction mixture was 115 µl and the reaction time 60 sec. The ^{125}I DFP-inactivated urokinase was purified on a small Sephadex G-25 column. Fifty to sixty per cent of the iodine was incorporated into the DFP-inactivated urokinase as calculated from the elution profile. This corresponds to a specific activity of 18–22 MBq/nmol.

Radioimmunoassay of urokinase was performed with a double antibody system. Each tube contained 0.2 ml antiserum diluted 1:20,000, 0.2 ml ^{125}I DFP-inactivated urokinase (approximately 0.3 ng) and 0.1 ml standard of urokinase or sample. The tubes were incubated at 4°C for two days. Then 0.1 ml normal human plasma, 0.05 ml of 1:250 diluted normal rabbit serum and 0.05 ml of 1:10 diluted goat antiserum to rabbit IgG were added. The tubes were mixed and incubated for another 4–18 hr at 4°C. The radioactivity of the precipitate was counted after centrifugation and decanting off the supernatant.

Organ culture

Fetal kidneys obtained at legal abortions or ovarian cystadenocarcinomas obtained at laparotomy were processed for organ culture. The specimens were divided into small explants of about 1 mm^3. The explants were then placed on gelatin sponge slices (Spongostan, Ferrosan, Malmö, Sweden) in plastic Petri dishes. A purely synthetic medium (Parker 199, 99 SBL, Stockholm) enriched with the tripeptide Gly-Lys-His [17] was then added. The cultures were run in 5% CO_2 in air for 44 days. Survival of the explants was checked by serial sectioning and histological examination. The medium was harvested every fourth day. The culture media from the cultures were constantly found to contain fibrinolytic activity when tested on plasminogen containing human fibrin plates [16] but not on plasminogen-free plates. The plasminogen activators in the culture medium of the fetal kidneys and ovarian carcinomas were partly purified by affinity chromatography on *para*-aminobenzamidine-CH-Sepharose followed by gel chromatography on Sephadex G-100 Superfine as described above for urokinase. Reference proteins for molecular estimations were: cytochrome C, soybean-trypsin inhibitor, chymotrypsinogen, ovalbumin, bovine serum albumin and phosphorylase-a read at 280 nm. As reference for assaying fibrinolytic activity urokinase (Ampouls à 10,000 Ploug units) from Löven, Copenhagen, was used.

RESULTS

The urokinase radioimmunoassay permitted the detection of purified urokinase down to 0.05 nmol with the present antiserum ($K_a = 2.5 \times 10^{-10}$) (Fig. 1). The ^{125}I-urokinase was rather stable, and could be used up to six weeks without further purification. It was bound to 70–75% by incubation with excess antibody. The intra-assay coefficient of variation was always less than 5% (mean of 66 determinations). The inter-assay coefficient of variation was 8%.

Assay of fetal kidney culture medium and ovarian tumour culture medium revealed concentrations of urokinase-like activity in concentrations ranging from about 0.5 to 43 nmol (Table 1). Assay of medium from cultures of normal human ovarian tissue did not detect any urokinase-like activity. The concentration

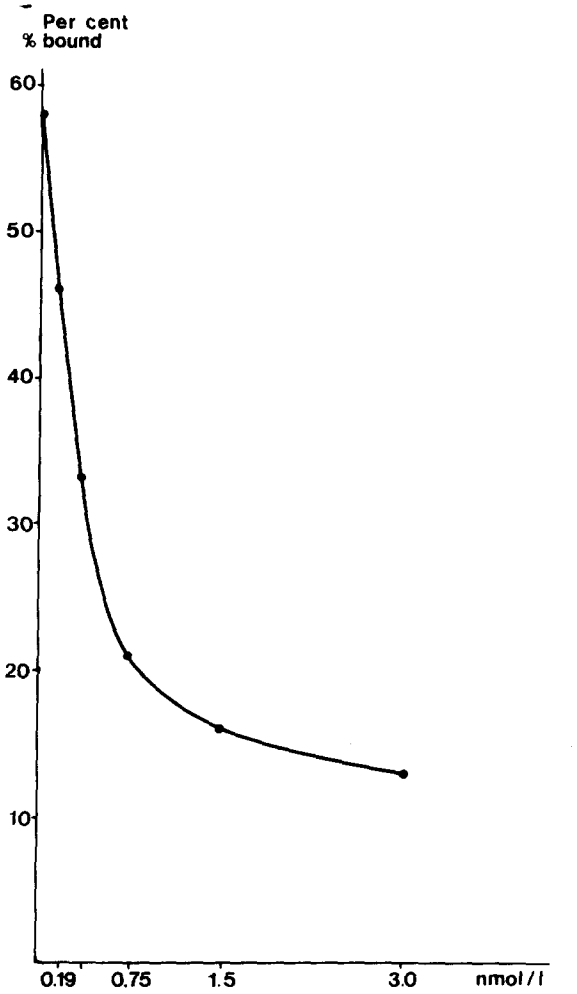

Fig. 1. Standard dose–response curve for urokinase determined by means of DFP-inactivated [^{125}I]-urokinase and rabbit antiserum to urokinase (urokinase of molecular weight 31,000 daltons was used throughout).

Table 1

Fetus	Fetal kidneys Early culture	Fetal kidneys Late culture		Ovarian cystadenocarcinomas Early culture	Ovarian cystadenocarcinomas Late culture
1	2.5	11.5	Serous	1.6	4.6
2	23.8	13.9	Serous	5.6	4.6
3	2.9	17.4	Serous	1.7	3.3
4	4.0	26.0	Serous	0.7	2.0
5	13.2	42.9	Serous	3.6	3.3
6	3.9	16.5	Mucinous	0.9	0.5
7	1.3	3.0	Mucinous	1.9	1.7

Concentration in nmol of antiurokinase-reacting material in early (1–2 weeks) and late (3–4 weeks) organ cultures of human fetal kidneys and human ovarian tumours.

was highest in the early period after explantation and decreased with time, but was still detectable after six weeks of culture. The duration of the culture period did not influence the slope of the inhibition curves as tested with crude media from tumour cultures assayed at various intervals after explantation (Fig. 2).

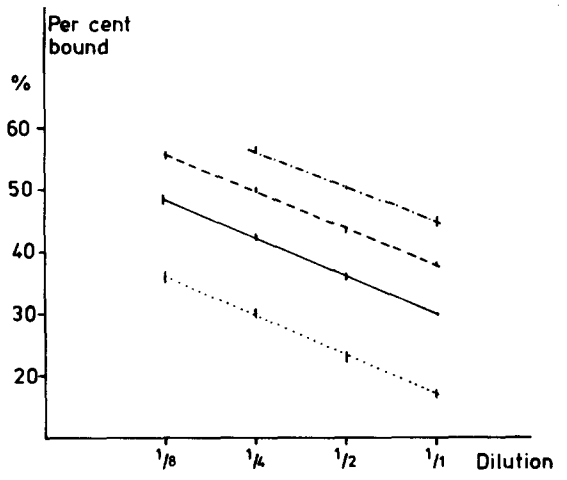

Fig. 2. Determination of antiurokinase-reacting plasminogen activator in crude medium from organ cultures of ovarian carcinoma after 8 ·····, 24 ---------, 44 -·-·-·-·- *days of culture. Urokinase standard* ————.

Gel chromatography on Sephadex G-100 Superfine of medium both from fetal kidney cultures and tumour cultures revealed three peaks of fibrinolytic activity, corresponding to molecular weights of about 100,000, 54,000 and 31,000 daltons (Fig. 3). In the radioimmunoassay the inhibition curves for these three fractions were parallel to the inhibition curve of the purified urokinase. The concentration of urokinase-like material in the fractions determined by the assay corresponded closely to their fibrinolytic activity. The correlation coefficient, $r=0.84$, $P<0.01$.

DISCUSSION

The development of the present radioimmunoassay for urokinase made it possible directly to quantify the release of urokinase-like immunoreactive material in culture media of fetal kidneys and ovarian tumours.

In the assay the inhibition curves of purified urokinase were parallel to the curves for each of the three fractions of immunoreactivity obtained at gel chromatography of fetal kidney and ovarian tumour culture medium. This indicates a close immunological cross-reaction between urokinase and the plasminogen activator released from fetal kidney and ovarian tumour explants. Determination of plasminogen activator in these chromatography fractions showed that the enzymatic activity had the same distribution as the immunoreactivity, which is in accord with observations in neutralization tests and double immunodiffusion experiments [11]. The immunological activity of the three forms correlated closely with their plasminogen activator activity. This indicates that the ratio of immunological and active sites in the various molecular forms are the same. Another support for this concept is the observation in this laboratory that three different forms of urokinase with different molecular weights (100,000, 54,000, 31,000) all contain the same 31,000 dalton protein chain in which the enzymatically active site is located and towards which the antiserum is directed (Åstedt *et al.*, unpublished results).

The gel filtration experiment demonstrates that the urokinase-like plasminogen activator released in ovarian tumour cultures occurs in the same molecular forms as urokinase [12, 18, 19] and the plasminogen activator released in kidney cultures [14, 20]. The three

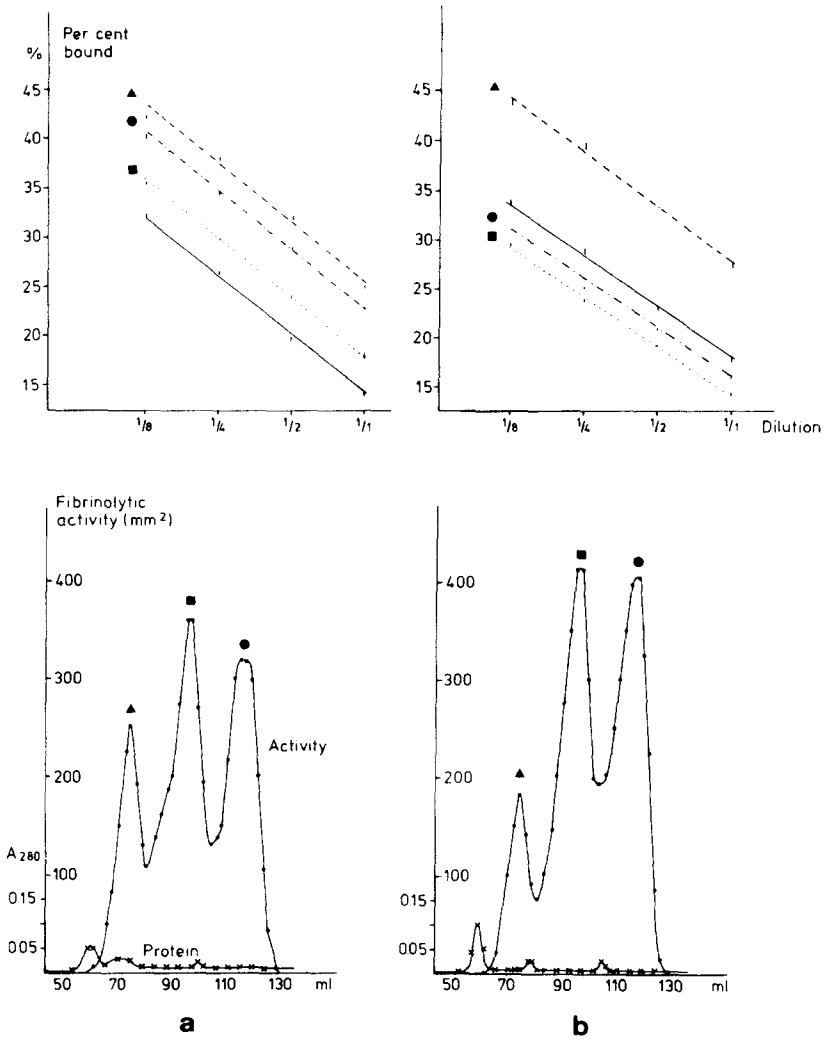

Fig. 3. Determination of antiurokinase reacting plasminogen activator by radioimmunoassay in organ culture media of fetal kidneys (a) and ovarian carcinomas (b). The plasminogen activators were partially purified on p-*aminobenzamidine-Sepharose and 3 peaks separated by gel filtration on Sephadex G-100 Superfine (see Materials and Methods) (below). The peaks corresponded to molecular weights of* 100,000 (▲), 54,000 (■) *and* 31,000 (●) daltons, *respectively. Each peak was analysed for antiurokinase reacting material (above).* ▲---100,000 dalton *peak,* ■····54,000 dalton *peak,* ●-·-·-·-31,000 dalton *peak.* ——*urokinase standard.*

molecular forms contained in the kidney and ovarian tumour cultures were similar as shown in the gel filtration experiment, and correspond to those of urokinase [12, 18, 19]. In cultures of fetal kidneys and ovarian tumours the relative proportions of the three forms vary greatly with the age of the culture [20, 21]. Distinct parallel lines in the radioimmunoassay were obtained by the three molecular forms as well as by crude medium from tumour cultures of varying age. This indicates that immunological determination of plasminogen activator in different samples will not be affected by varying proportions of the molecular forms.

Urokinase and the tumour plasminogen activator are both inactivated by diisopropylfluorophosphate, which shows that they are serine proteases [11]. Thus, the tumour plasminogen activator and urokinase are similar, if not identical, not only in respect of molecular weights and immunologic determinants but also of active sites.

Previously it had not been possible to measure the release of plasminogen activators from malignant ovarian tumours *in vitro* by

direct methods. Indirect methods have been necessary, such as introduction of a plasminogen-contaminated fibrin clot in the culture tube and measurement of the accumulation of fibrin degradation products in the medium [7]. The assay described here facilitates such tissue culture studies by giving the molar concentrations of the plasminogen activators released.

In vivo, the fibrinolytic activity of human ovarian tumours results in the occurrence of fibrinogen/fibrin degradation products in ascitic fluid [22] and in the blood [23, 24]. However, the results of determinations of such products in the blood have been variable and of limited diagnostic value. Direct detection of plasminogen activators released from ovarian tumours *in vivo* has not yet been possible. If interference with naturally occurring inhibitors can be avoided, the improved sensitivity of the radioimmunoassay presented here might prove useful for the detection of tumour plasminogen activator in peripheral blood.

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