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Human tissue-type plasminogen activator

Production in continuous serum-free cell culture and rapid purification

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A simplified procedure for the production and purification of human tissue-type plasminogen activator (t-PA) is described. Bowes-melanoma cells were maintained in continuous serum-free culture. The cell nutrient consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with insulin (5 mg/litre), transferrin (5 mg/litre), progesterone (1 nM), cortisol (10 nM), aprotinin (2×10^4 units/litre) and a mixture of trace elements. t-PA accumulated in the culture medium at a rate of 40 units/day per ml and was harvested every third day. Cell losses during each harvest, leading to a steady decline of enzyme yields, were compensated for by treating the cells with 5% (v/v) fetal-bovine serum in DMEM every 6–8 weeks. t-PA was rapidly purified by a combination of cation-exchange chromatography and gel filtration. The procedure yielded mainly single-chain t-PA of a specific activity of 80000 to 100000 units/mg.

Thrombolysis as a therapeutic approach to remove enzymically blood clots in the vascular system has recently gained renewed interest. Thromboembolic events are a common cause of life-threatening diseases such as pulmonary embolism, acute myocardial infarction and stroke. Suitable thrombolytic agents activate the plasma zymogen plasminogen to plasmin (EC 3.4.21.7), a potent fibrin-degrading enzyme. Human tissue-type plasminogen activator (t-PA) achieves thrombolysis without concomitant systemic alterations in fibrinogen and α_2-antiplasmin levels (Matsuo *et al.*, 1981; Korninger *et al.*, 1982). Thus the dangerous bleeding tendency induced by streptokinase or urokinase may be avoided. The scarcity of t-PA has limited the scale of controlled clinical studies. Therefore progress in this field is dependent on the development of economical methods for the production of t-PA.

Below we report conditions for the production of t-PA in continuous serum-free cell culture and a simple rapid purification procedure.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulphate; t-PA, tissue-type plasminogen activator; FBS, fetal-bovine serum; SP-Sephadex, sulphopropyl-Sephadex; SF medium, serum-free medium (see the text for full composition); phosphate-buffered saline, pH 7.3 buffer of composition (g/litre): NaCl, 8; KCl, 0.2; Na_2HPO_4, $2H_2O$), 1.44; KH_2PO_4, 0.2.

Experimental

Materials

Dextran (Macrodex), protein A–Sepharose, SP-Sephadex and M_r-marker proteins phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000) and carbonic anhydrase (30000) were from Pharmacia, Uppsala, Sweden; fibrinogen (bovine) from Poviet, Oss, The Netherlands; thrombin (Topostasin) from Hoffmann–LaRoche, Basel, Switzerland; insulin and transferrin from Calbiochem, La Jolla, CA, U.S.A.; progesterone (Lutocyclin) from Ciba–Geigy, Basel, Switzerland; cortisol (Solu-Cortef) from Upjohn, Puurs, Belgium; aprotinin (Trasylol) from Bayer, Leverkusen, Germany; the Immun-Blot assay kit and all reagents for SDS/polyacrylamide-gel electrophoresis from Bio-Rad, Richmond, CA, U.S.A.; tissue-culture flasks from Corning Glass Works, Corning, NY, U.S.A., and the cell factories (Multitray Units, 335 mm × 205 mm × 190 mm) from Nunc, Roskilde, Denmark. Bowes-melanoma cells were obtained from Professor Daniel B. Rifkin, Department of Cell Biology, New York University, NY, U.S.A.

Assay of t-PA activity

A 30 μl portion of a suitably diluted sample was added to 0.25 ml of a solution containing fibrinogen (1.5 mg/ml), plasminogen (50 μg/ml) and dextran (15 mg/ml) in 0.15 M-Tris/HCl, pH 7.8, prewarmed to 37°C. After 1 min, 50 μl of thrombin (10 units/ml) were added. The lysis times (interval between addition of the thrombin and complete lysis of the clot) were compared with those obtained by using a laboratory-standard t-PA preparation [1 unit/ml corresponds to a t-PA concentration of 10 ng/ml]. [In June 1984, the Expert Committee of Biological Standardization of the World Health Organisation established an International Standard for human t-PA. Comparison with our laboratory standard t-PA preparation revealed that one of our units corresponds to 3.4 i.u. when assayed on a conventional fibrin plate]. t-PA concentrations between 25 and 200 units/ml yielded lysis times between 20 and 7 min and correlated with the reciprocal of lysis time squared. Protein was quantified by the method of Bradford (1976), with bovine serum albumin as a standard. Protein determinations in culture supernatant were made with albumin standards in DMEM.

Cell culture

Bowes-melanoma cells (Rifkin *et al.*, 1974) were cultured in DMEM supplemented with 5% FBS or with insulin (5 mg/litre), transferrin (5 mg/litre), progesterone (1 nM), cortisol (10 nM) (Mather & Sato, 1979), aprotinin (2×10^4 units/litre) and a mixture of trace elements (Hutchins & Sato, 1978) (SF medium).

Antibodies against human t-PA

Human t-PA was purified from the conditioned media of 12-*O*-tetradecanoyl phorbol-13-acetate stimulated HeLa cells and injected into rabbits for the generation of antibodies. IgG was isolated by affinity chromatography on protein A–Sepharose. It precipitated a single [^{35}S]methionine-labelled protein from conditioned medium of HeLa cells or of Bowes-melanoma cells (Fig. 1). Details of these procedures will be described elsewhere (Waller & Schleuning, 1985).

Electrophoretic procedures

SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) was performed in a 12% (w/v) gel. Proteins were electroblotted from the gels to nitrocellulose and revealed by immunochemical staining (Towbin *et al.*, 1979) using rabbit anti-(human t-PA) IgG followed by goat anti-rabbit IgG–horseradish peroxidase conjugate provided with the Bio-Rad Immun-Blot assay kit.

Electrophoretic–zymographic analysis of t-PA on fibrin–agarose underlays was done by the method of Granelli-Piperno & Reich (1978) as modified by Tissot *et al.* (1982).

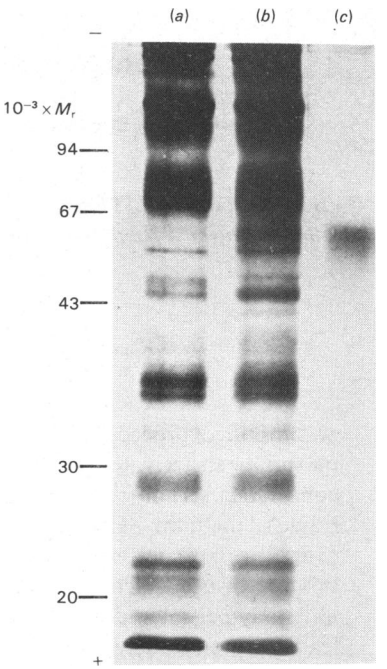

Fig. 1. *Characterization of anti-t-PA antibody specificity*
A 5 ml portion of metabolically [^{35}S]methionine-labelled conditioned medium of Bowes-melanoma cells was passed over a 1 ml column of anti-t-PA IgG immobilized on CNBr-activated Sepharose 4B. After washing the column with 100 ml of phosphate-buffered saline containing 0.5 M-NaCl/0.002 M-EDTA/0.1% Tween 80 and 0.01% SDS, t-PA was eluted with phosphate-buffered saline/3 M-NaSCN, containing horse myoglobin (200 μg/ml) to prevent adsorptive losses of t-PA. Lanes (*a*) and (*b*) represent the labelled conditioned medium after (*a*) and before (*b*) passage through anti-t-PA IgG–Sepharose. After dialysis, the immune eluate contained 600 t-PA units of plasminogen activator activity/ml; it moved as a single band of M_r 67000 on SDS/polyacrylamide-gel electrophoresis (lane *c*).

Results

Preliminary experiments had shown that melanoma cells were well maintained in SF medium, but that their growth rate was extremely slow. Therefore, for large-scale production of t-PA, melanoma cells were first grown to confluency in DME containing 5% FBS in 6000 cm^2 Nunc cell factories. After the cells reached confluency, the factories were washed twice with phosphate-buffered saline (1 litre per factory) and incubated

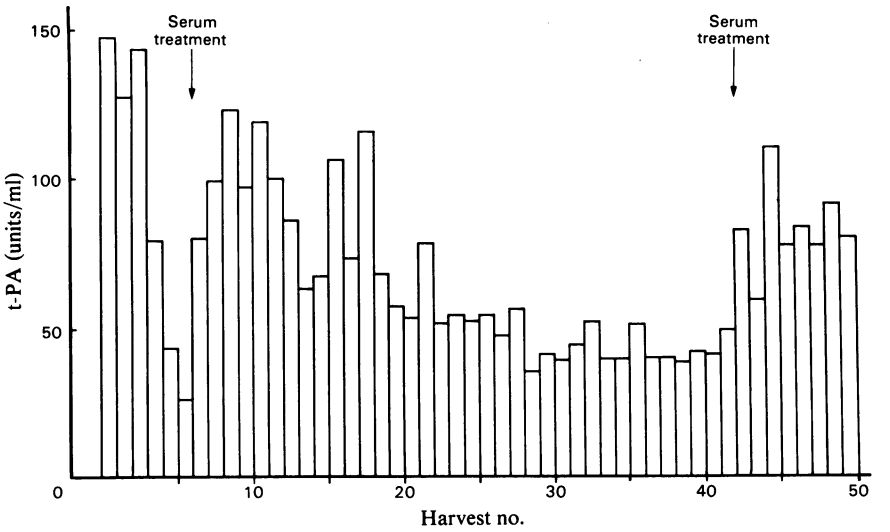

Fig. 2. *Large-scale continuous production of t-PA in SF medium*
Melanoma cells were grown to confluency in four cell factories in DMEM + 5% FBS, twice washed with phosphate-buffered saline and incubated for 6h in DMEM. Each factory was then filled with 1 litre of SF medium which was replaced every 3–4 days. At the times indicated by arrows, the cells were treated for 2 days with DMEM + 5% FBS, twice washed with phosphate-buffered saline, incubated for 6h in DMEM and again cultivated in SF medium.

for 6h in DMEM alone to remove the bulk of the serum proteins. The cells were then further incubated in SF medium (1 litre/factory). The medium was replaced every 3–4 days. Fig. 2 shows t-PA activity in the conditioned medium for 50 subsequent harvests. t-PA activities tended to decline with time owing to losses of cells from the factory, but could be restored by a 3-day treatment of the cells with DMEM + 5% FBS. With interruption by serum treatment every 6–8 weeks, the same production rate could be maintained for over 8 months.

Purification of t-PA

The conditioned medium was centrifuged at 20°C and 2000*g* for 10min. Tween 80 (0.01%) and NaN_3 (0.1%) were added to the supernatant. This was stored at 4°C for up to 4 days. The pH was adjusted to 4.5 by dropwise addition of 6M-HCl. A fine precipitate was removed by centrifugation (30min, 4°C, 4000*g*). Fig. 3 demonstrates the ion-exchange purification step with SP-Sephadex at pH4.5. Most of the contaminating proteins passed unadsorbed through the column (result not shown). As judged from activity measurements in the run-through fractions, t-PA bound quantitatively to the gel. Eluate fractions with activities above 1000 units/ml were pooled, concentrated to 7ml in an Amicon stirred cell with a YM 30 membrane, and passed over a 600ml column of Sephadex G-100

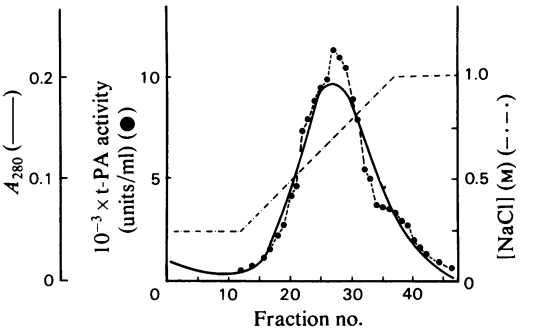

Fig. 3. *Purification of human t-PA on SP-Sephadex*
Melanoma culture supernatant (6.5 litres) was adjusted to pH4.5 and passed at a rate of 500ml/h over a 3.2cm × 10cm (80ml) SP-Sephadex column previously equilibrated with 0.1M-sodium acetate/0.25M-NaCl/0.01% Tween 80/0.05% NaN_3, pH4.5. The run-through fractions contained about 280mg of protein (not shown), but less than 1% of total activity. After washing of the column with 150ml of the same buffer, t-PA was eluted by a NaCl gradient from 0.25 to 1M in the same buffer. The activity of 6ml fractions was measured by using the fibrin-clot-lysis assay (●). The concentration of NaCl was determined with a conductivity meter (-·-·); A_{280} (——) was also measured.

(Fig. 4). t-PA activity was almost quantitatively eluted with the second protein peak. Fractions with activities above 1000 units/ml were com-

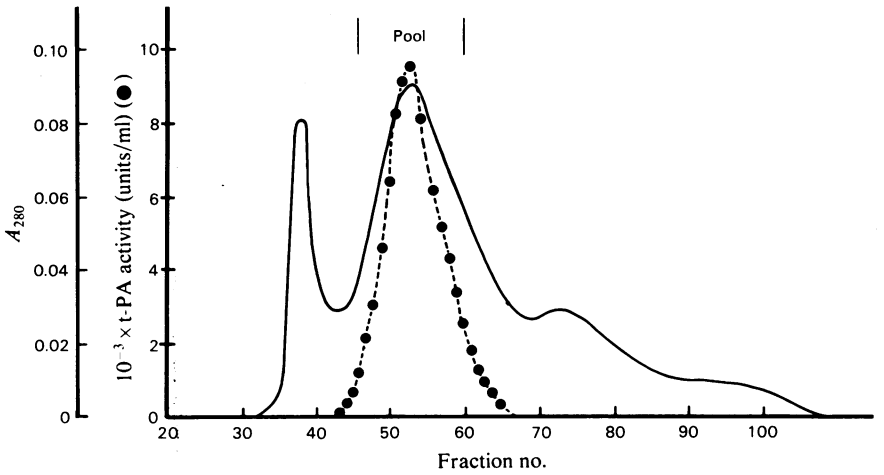

Fig. 4. *Purification of human t-PA on Sephadex G-100*
Peak activity fractions (80ml) of the SP-Sephadex step were pooled, concentrated to 7ml by ultrafiltration and passed over a 3.2cm × 75cm (600ml) Sephadex G-100 column in 50mM-sodium acetate/0.5M-NaCl/0.01% Tween 80/0.05% NaN_3, pH4.5. The activity of 5ml fractions was measured by using the fibrin-clot-lysis assay (●); A_{280} (——) was also measured.

Table 1. *Purification of human t-PA from melanoma culture supernatant*

	Volume (ml)	[Protein] (mg/ml)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Culture supernatant	6500	0.045	71	460000	1600	1	100
SP-Sephadex after concentration	7	0.93	42800	300000	46000	29	65
Sephadex G-100 after concentration	8	0.38	35000	280000	92000	58	61

bined, concentrated by ultrafiltration in the stirred cell and stored at −70°C. This preparation had a specific activity between 80000 and 100000 units/mg (Table 1).

Characterization of purified t-PA

The purified material contained mainly single-chain t-PA. This is illustrated in Fig. 5. Protein staining of non-reduced (lane 1) or reduced (lane 2) samples as well as immunoblotting of non-reduced (lane 3) or reduced (lane 4) material, and zymographic detection of plasminogen activator activity on plasminogen-rich fibrin–agarose underlays consistently revealed a major band in the 60000–67000-M_r region. The M_r obtained for reduced t-PA was about 67000, a value which is similar to that found by others (Rijken & Collen, 1981; Wallén *et al.*, 1983). In reduced samples, several additional bands were seen on stained gels, most of which reacted with anti-t-PA IgG as revealed by immunoblotting. The doublet in the 60000-M_r region (lanes 2 and 4) corresponds to a slightly degraded single-chain t-PA (Bányai *et al.*, 1983) exhibiting also some fibrinolytic activity (lane 5); the bands in the 35000–38000-M_r region correspond to variably glycosylated forms of the A-chain (reviewed by Bachmann & Kruithof, 1984), and the 32000-M_r form is attributable to the B-chain of double-chain t-PA. Lanes 2 and 4 demonstrate that one further minor band of M_r 24000 is t-PA-related. Another minor band of M_r 31000 apparently represents a contaminating protein, since it was only present on stained gels.

Discussion

In animal models of thrombosis and myocardial infarction, t-PA was shown to be a thrombolytic agent superior to urokinase and streptokinase

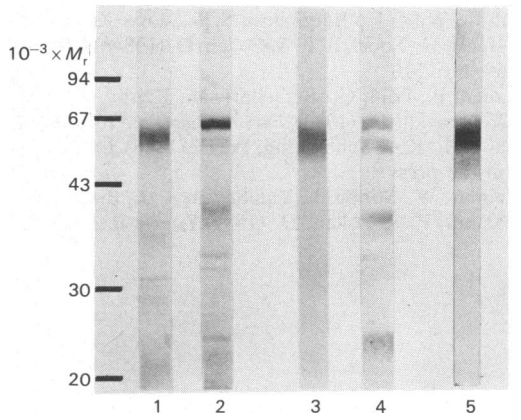

Fig. 5. *Electrophoretic analysis of purified human t-PA*
SDS/polyacrylamide-gel-electrophoretic patterns in a 12% (w/v) gel. 1 and 2, Coomassie Brilliant Blue-stained gels of 20 μg of non-reduced (1) and reduced (2) t-PA; 3 and 4, peroxidase-stained bands of 1 μg of non-reduced (3) and reduced (4) t-PA, electrophoretically transferred after SDS/polyacrylamide-gel electrophoresis to nitrocellulose and incubated with rabbit anti-t-PA antibodies followed by goat anti-rabbit IgG–horseradish peroxidase conjugate; 5, zymographic analysis of 2.5 μg of purified non-reduced t-PA after 3h incubation on a fibrin–agarose underlay.

(Korninger *et al.*, 1982; Mattsson *et al.*, 1983; Collen *et al.*, 1983; Bergmann *et al.*, 1983). In man, thrombolytic therapy with t-PA has been successful (Weimar *et al.*, 1981; Van de Werf *et al.*, 1984*a*). Therefore production of large quantities of t-PA at reasonable cost is desirable. t-PA has been previously purified from the culture supernatant of Bowes-melanoma cells (Rijken & Collen, 1981; Wallén *et al.*, 1983). The large-scale production and purification of t-PA by the described methods, however, is costly and time-consuming. The production of t-PA by recombinant-DNA technology (Pennica *et al.*, 1983; Edlund *et al.*, 1983) may make available large quantities of t-PA in the near future, but it remains to be established whether t-PA produced in bacteria or yeast exhibits the same behaviour *in vivo* as native t-PA. The production of t-PA by recombinant-DNA technology in mammalian cells (Van de Werf *et al.*, 1984*b*) after gene transfection and amplification is advantageous in terms of enzyme yields, but the cells have to be cultivated in the presence of serum. This increases media costs and renders purification more difficult. The continuous production of t-PA by melanoma cells in serum-free medium described here greatly reduces the investment of labour and the cost of media. Compared with the supplements recommended by Hutchins & Sato (1978) and Mather & Sato (1979), we restricted the number of hormones to the minimum necessary for maintaining t-PA production. Some cell losses, however, occurred during media changes, which made it necessary to treat the cells every 6–8 weeks with serum containing medium to restore the original cell density. The absence of serum-derived proteins greatly facilitated the purification of t-PA from the conditioned medium. The simple two-step purification procedure is reproducible and can easily be scaled up. The final product consisted mainly of single-chain t-PA, but minor quantities of a slightly degraded single-chain form of t-PA (Bányai *et al.*, 1983) and of double-chain t-PA were present.

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