Volume 238, number 1, 129–134 FEB 06346 September 1988

Heterogeneity of human tissue-type plasminogen activator

Ghislain Opdenakker, Fons Bosman, Benny Decock, Yofre Cabeza-Arvelaiz, Jo Van Damme and Alfons Billiau

Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Received 20 July 1988

Tissue-type plasminogen activator (t-PA) from human melanoma cells (Bowes) was purified by immunosorbent chromatography on affinospecific polyclonal antibodies and gel filtration in the presence of KSCN. The immunosorbent eluate contained three major components of >200, 85 and 65 kDa, respectively. The 65 kDa t-PA component could be separated by gel filtration on Ultrogel AcA44 in the presence of KSCN to a pure preparation yielding a unique N-terminal amino acid sequence. Immunoblot analysis, using affinospecific antibodies against t-PA, was a specific and sensitive method to identify different types of t-PA (I–IV), as well as t-PA-inhibitor complexes and degradation products in unstimulated melanoma cell culture fluids. Furthermore, the t-PA preparations, produced by phorbol ester-treated melanoma cells, were free of type IV and thus differed physicochemically from the constitutively produced t-PA preparations. The composition of t-PA from mammalian cell cultures is thus more complex than hitherto described.

Tissue-type plasminogen activator; Heterogeneity; Tumor promotion; Protein processing; Electrophoresis

1. INTRODUCTION

Plasminogen activators are serine proteases, which are involved in various physiological and pathological processes, which require the degradation of macromolecules: fibrinolysis, tissue-remodelling and cell migration in organogenesis, inflammation and perhaps also in malignant invasion and metastasis [1,2]. In the human species there exist at least two major different types of plasminogen activators: urokinase (u-PA) [3] and tissue-type plasminogen activator (t-PA) [4]. They are the products of different but related, spliced, single-copy genes located on the human chromosomes 10 and 8, respectively [5–8].

Because of its potential use in the therapy of thromboembolic diseases, t-PA has recently received much attention both in fundamental and applied studies [9–12]. Several studies indicate that not only the polypeptide structure, but also the carbohydrate chains might influence the specific activities of t-PA molecules [13–15]. Interactions of t-PA with specific inhibitors [16,17] and fibrin [18] have been described.

After the initial purification of t-PA from uterine tissue [19], the human melanoma cell line (Bowes) has been widely used to purify t-PA by affinity chromatography on Zn-chelate and Con A-Sepharose [20], on polyclonal [21] and monoclonal [22] antibodies and on specific inhibitors such as the trypsin inhibitor of *Erythrina latissima* [23]. The same cell line has originally been used to isolate t-PA mRNA [24] for cDNA cloning experiments [4,25–29]. The complete gene as well as its flanking regions have been sequenced [30].

Several types of heterogeneity have been described for t-PA: single- and two-chain forms, N-terminus sequence heterogeneity seemingly associated with a microheterogeneity in protein sequence [31], and glycosylation pattern differences, resulting in the appearance of type I and II variants, separable by SDS-PAGE [14].

In the present study we used Western-blot analysis to show the existence of at least 2 additional types (III and IV) of t-PA in the culture fluids of a human melanoma cell line and to il-

Correspondence address: G. Opdenakker, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

lustrate that the relative amounts of these different types can change under specific culture conditions.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The human melanoma cell line (Bowes, RPMI 7272) was made mycoplasm-free by inoculation in nude mice and transfer of cells from the three-week solid tumor to in vitro culture. The cells were propagated and treated with the tumor promoting phorbol-myristate-acetate as described before [32–34]. Cell culture fluids were collected as serum-free medium containing 25 kallikrein-inhibitory units of aprotinin per ml.

2.2. *Preparation of affinospecific antibodies against human melanoma t-PA*

Melanoma t-PA was purified to homogeneity as described [20]. Polyclonal antibodies were raised in rabbits by repeated injections of 10 μg of t-PA, emulsified in complete Freund's adjuvant. Then booster injections were given at two week intervals. One week after the 5th booster 20 ml of peripheral blood was isolated. The immunoglobulin fraction was precipitated with ammonium sulphate and the IgG fraction was isolated by affinity chromatography on protein A-Sepharose (Pharmacia). Electrophoretically pure t-PA (200 μg) was immobilized to CNBr-activated Sepharose CL-4C (Pharmacia). The IgG preparations were then affinity-purified and finally neutralized and concentrated by dialysis against 20 mM phosphate buffer, pH 7.2, 0.5 M NaCl, 5% polyethyleneglycol.

2.3. *Purification of t-PA by immunosorbent and gel-filtration chromatography*

Affinospecific antibodies against t-PA (2.5 mg) were coupled to CNBr-activated Sepharose CL-4B. The gel was equilibrated with phosphate buffered saline, supplemented with 2 M NaCl, 0.01% Tween 80, and 25 units of aprotinin per ml. Approx. 500 ml of serum-free melanoma culture fluid was chromatographed. The column was sequentially washed with the equilibration buffer: 1 M NaCl, 0.1 M glycine, NaOH, pH 9.5, and 20 mM phosphate buffer, pH 7.2. The t-PA was desorbed with 3.5 M KSCN. All fractions containing amidolytic activity were concentrated by dialysis and applied to Ultrogel AcA44, equilibrated with 20 mM phosphate buffer, pH 7.2, 1.6 M KSCN.

2.4. *Protein determination, amidolytic assays, fibrin agar zymography, and amino acid sequence analysis*

Protein concentration was measured as described [35] using bovine serum albumin as a protein standard. Amidolytic activities were determined with a sensitive automated micromethod and expressed as described [13]. Zymography of plasminogen activators was as described [36]. The N-terminal sequence of purified unmodified t-PA was determined with a 477A sequenator with on-line detection of the PTH residues in a 120A analyser (Applied Biosystems Inc.).

2.5. *Electrophoresis and immunoblot analysis*

Protein samples were separated in vertical polyacrylamide slab gels in the presence of 0.1% SDS [37]. Fixed proteins in the gels were stained with Coomassie brilliant blue. For immunoblot analysis, electrophoretically separated proteins were transferred onto nitrocellulose membranes, using a semi-dry electroblot apparatus (Jancos, Denmark) [38]. After overnight reaction of the membrane-bound proteins with the affinospecific anti-t-PA antibodies, the membranes were extensively washed and then exposed to pig, peroxidase-labelled, anti-rabbit IgGs (Dakopatts, Denmark). Finally the membranes were washed and reacted with H_2O_2.

3. RESULTS

3.1. *Purification of t-PA*

Approx. 500 ml of serum-free culture fluid from unstimulated or phorbol ester-treated melanoma cells was subjected to affinity chromatography on affinospecific antibodies directed against t-PA. Desorption of the bound molecules was monitored by electrophoretic analysis under reducing conditions and protein staining. Three major protein bands with apparent M_r values of >200000, 85000 and 65000, respectively, were visualized in the eluted fractions, which contained most of the total applied amidolytic activity. The enzymatically active fractions of the immunosorbent column were pooled, concentrated and subjected to gel filtration. Two major peaks were obtained of which only the second contained amidolytic activity. Analysis by SDS-PAGE under non-reducing conditions and protein visualization by Coomassie blue staining showed the presence of two not sharply delineated bands (t-PA types I and II, arrowheads fig.1, migrating close to each other in the 65–70 kDa region) in the fraction containing the amidolytic activity and comigrating with bovine serum albumin. Zymographic analysis by the fibrin agar overlay technique showed that these protein bands coincided with the fibrinolytic activity (fig.1). A urokinase control sample showed on zymography, as expected, the presence of two proteins with plasminogen activating properties (55 kDa and an autocatalytical cleavage product of 33 kDa). This urokinase sample appeared as a single protein band of 55 kDa after SDS-PAGE and staining. The appearance of the lysis zones was dependent on the presence of plasminogen (not shown). The overall yields and the purification factors of the two-step purification method are summarized in table 1. Approx. 10 μg of the purified product was subjected to sequential degradation in a protein microsequencer. The unique sequence obtained was Ser-Tyr-Gln-Val-Ile-Xaa-Arg-Asp-Glu-Lys... This sequence is identical with that

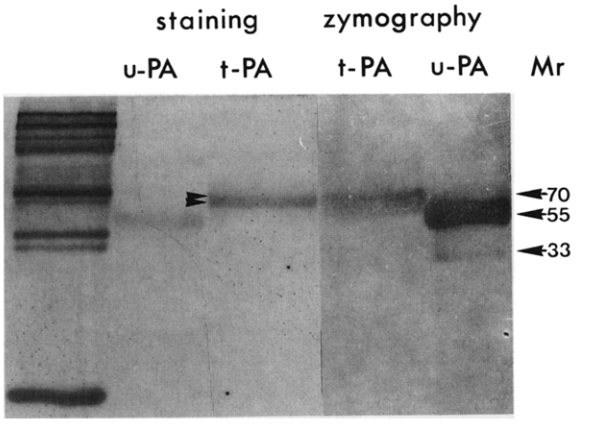

Fig.1. Analysis of purified t-PA. The purified t-PA (10 μg) and a control sample of u-PA were subjected to SDS-PAGE and the proteins were visualized by Coomassie brilliant blue staining (left). Samples both of t-PA and u-PA were run in parallel and after separation the plasminogen activators were developed by fibrin agar zymography in the presence of plasminogen (right). Arrowheads indicate the doublet types I and II of t-PA. Apparent molecular masses are indicated in kDa.

previously described for the N-terminus of t-PA, where the Xaa position corresponds to a cysteine residue, not detectable in unmodified samples. This analysis furthermore corroborates that the obtained product is not contaminated with other N-terminal unblocked proteins.

3.2. *Immunoblot analysis*

Batches of 500 ml serum-free culture supernatants from melanoma cells, treated with 100 ng/ml of phorbol ester or from untreated cells were purified as described and analyzed by Western-blot analysis using the affinity-purified polyclonal antibody. Surprisingly, cell culture fluids from unstimulated Bowes melanoma cells contained not only types I and II, that have been previously described, but also two additional t-PA species, henceforth called types III and IV. These new types of t-PA, when compared to types I and II, migrated faster and occurred as a less intense doublet with an apparent M_r of about 60000. Types I–IV were reproducibly observed in our, as well as in other preparations of t-PA (fig.2). Moreover the described Western-blot technique was useful to detect t-PA-inhibitor complexes and degradation products. Such complexes have previously been described to be resistant to dissociation under the used conditions of analysis [16]. The immublot technique is a specific and sensitive assay for t-PA in that samples containing less than 10 ng of t-PA yielded the 4 different types. Direct protein visualization by Coomassie blue staining is not suited to detect these minor variants and is at least 100 times less sensitive than Western blot analysis [14]. With the use of our affinity-purified antiserum we were able to show a hitherto

Table 1

Purification of human t-PA from melanoma cells

Purification step	Total amount of protein[a]		Yield[b] (%)	Purification factor
	(μg)	(%)		
Culture medium	20100[c]	100	–	1
Immunosorbent	138	0.81	73	106
Ultrogel AcA44	85	0.49	65	151

[a] The total amount of protein from 450 ml culture fluid was determined by the Bradford protein assay using BSA as a reference protein

[b] Recovery yields as determined by the chromogenic substrate release assay

[c] Each individual value is the mean of three independent experiments with 450 ml culture fluid (SE < 10%)

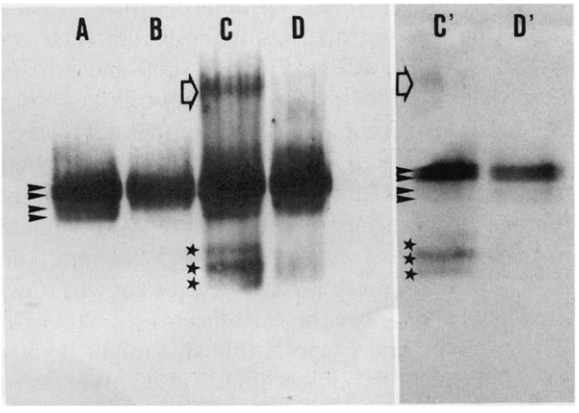

Fig.2. Immunoblot analysis of t-PA preparations. Nanogram quantities of melanoma cell t-PA, purified as described in section 2 were separated by SDS-PAGE, transferred to nitrocellulose and stained by the peroxidase-reaction. The closed arrowheads indicate the four types I–IV, the open arrow and the asterisks indicate the complex and the degradation products, respectively. Lanes were loaded as follows: unstimulated t-PA (lane A), phorbol-ester-induced t-PA (lane B), commercial t-PA (American Diagnostica, lane C) and t-PA as in lane A after 3 cycles of freezing/thawing (lane D). Lanes C′ and D′ are loaded with <10% of the material as in lanes C and A, respectively.

undetected qualitative difference in t-PA preparations from the same cell line under the pressure of a biological response modifier. From fig.2 (lanes A and B) it is clear that type IV t-PA is undetectable in the culture supernatants from phorbol ester-treated melanoma cells, whereas in a similar untreated culture this species was clearly identifiable.

4. DISCUSSION

Three major conclusions can be drawn from the present study: (i) the described purification technique (affinity and gel-filtration chromatography) yields t-PA preparations devoid of t-PA complexing activities and t-PA degradation products. The obtained product is N-terminus homogeneous and has a single-chain nature. (ii) The immunoblot analysis of purified t-PAs is at least 100 times more sensitive than Coomassie blue protein staining techniques and reveals novel species of t-PA in biological samples; and (iii) phorbol ester treatment of Bowes melanoma cells results in the secretion of only 3 out of 4 types of t-PA, indicating that physicochemically different t-PA preparations are obtained from the same cell line under different (patho)physiological conditions.

The immunosorbent chromatography yielded three major protein bands in the eluate with apparent M_r values of >200000, 85000 and 65000, respectively. To investigate if the two largest protein bands also contained t-PA peptides complexed to other molecules (which might explain their binding to the affinity reagent), we performed an immunoblot analysis of the mixture of the eluate molecules. In this case, only the >200 and the 65 kDa proteins were detectable (not shown). One other study describes the existence of a >200 kDa t-PA complex and suggests that this might be a t-PA-α_2-macroglobulin complex [39]. We have therefore purified this >200 kDa protein to homogeneity and probed it with a monoclonal antibody directed against human α_2-macroglobulin in a radio-immuno-assay. No α_2-macroglobulin antigenicity could be detected either in the unpurified or in the purified >200 kDa protein preparations (Professor F. Van Leuven, personal communication). The nature of the 85 kDa protein, unrelated to t-PA, was not further investigated.

The presence of KSCN during the gel-filtration chromatography seems to be crucial to separate the three components of the affinity eluates. Gel filtration in the absence of KSCN, FPLC ion exchange on mono Q, as well as affinity chromatography on arginine-Sepharose CL-4B proved to be inadequate methods to purify t-PA.

The purified material was analyzed by protein staining and zymographic analysis after electrophoretic separation. Urokinase was used as a control sample. Coomassie brilliant blue staining showed that t-PA migrated as two, not sharply delineated, protein bands, whereas u-PA could be visualized as one unsharp band. Renaturation of the separated proteins and fibrin agar overlay zymography could develop t-PA activity as one broad band, tending to lyse the polymerized fibrin also in the region of proteins migrating faster than those visualized with the direct staining technique. The u-PA sample developed fibrinolytic activity coinciding with the stainable protein, but also with the autocatalytic u-PA fragment of 33 kDa.

When purified t-PA samples of different sources were analyzed by immunoblotting, using the affinity-purified polyclonal antibody, two novel types of t-PA were found (types III and IV). These constitute most probably less than 10% of the total amount, since they were not visualized by protein staining and also because they were detected as less intense bands by the immuno-peroxidase reaction. More remarkable is the observation that commercially available preparations of t-PA seem to contain t-PAs complexed to other molecules as well as t-PA degradation products. Such complexes were also observed in our preparations of t-PAs of lesser purity (e.g. >200 kDa complex). The observation of more than two types of t-PA in culture fluids of eukaryotic cells is not restricted to the melanoma cell line but seems to be a universal characteristic of t-PAs secreted by mammalian cell cultures, both untransfected and transfected with the t-PA gene (in preparation). This implies that t-PA, generated by recombinant DNA technology is a more complex and heterogeneous mixture than previously thought. From earlier studies [13,14] it is clear that different types of t-PA have different specific activities. Purification of all types and detailed structural and functional analysis will be needed to define these differences between the various t-PA species. Possible explanations for the existence of structural and functional variants are: changes in glycosylation, N-terminal, C-terminal

or internal clipping or other posttranslational modifications.

Another interesting finding of our studies is the observation of differences in the relative composition of t-PAs, derived from the same cell substrate, but produced under different conditions. We have previously shown that the expression of the t-PA gene is enhanced in the melanoma cell line by phorbol esters [13,32–34]. A careful analysis of the genomic sequence of the t-PA gene [30] showed the presence of five AP-1 sites (at positions −3321, −2316, 11189, 15360, and 16999, respectively). These sites, with the recognition sequence TGACTCA, are the *cis*-acting regulatory elements that confer tumor promoter-mediated inducibility to different genes [40]. More recently it was found that the same consensus sequence functions as a recognition point of the activated jun-oncogene product, suggestedly identical to the transcription factor AP-1 [41]. The observation that after phorbol ester treatment melanoma cells fail to produce type IV t-PA implies that such cells contain differently processed t-PA proteins when compared to untreated cells. Alternative splicing or abnormal termination of transcription seems not to occur, since no mRNAs, abnormal in size, are observed on Northern blot analysis. Nevertheless, the absence of type IV illustrates that there exists a physicochemical difference between uninduced and phorbol ester-induced t-PA preparations.

Acknowledgements: The authors thank Professor T.W.R. Rademacher (Oxford University) and Professor K. Danø (Finsen Laboratory, Copenhagen) for helpful discussions. The present study was supported by the Cancer Research Foundation of the Belgian General Savings and Retirement Fund (ASLK). G.O. and J.V.D. are research associates of the Belgian National Fund for Scientific Research. Y.C.A. is a fellow of the Rega Foundation. The excellent assistance of P. Fiten is greatly acknowledged.

REFERENCES

[1] Danø, K., Andreasen, P., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L. and Skriver (1985) Adv. Cancer Res. 44, 139–266.
[2] Reich, E. (1975) in: Proteases and Biological Control (Reich, E. et al. eds) pp.333–341, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[3] Verde, P., Stoppelli, M., Galeffi, P., Di Nocera, P. and Blasi, F. (1984) Proc. Natl. Acad. Sci. USA 81, 4727–4731.
[4] Pennica, D., Holmes, W., Kohr, W., Harkins, R., Vehar, G., Ward, C., Bennett, W., Yelverton, E., Seeburg, E., Heyneker, H., Goeddel, D. and Collen, D. (1983) Nature 301, 214–221.
[5] Benham, J., Spurr, N., Povey, S., Brinton, B., Goodfellow, P., Solomon, E. and Harris, T. (1984) Mol. Biol. Med. 2, 251–259.
[6] Tripputi, P., Blasi, F., Verde, L., Cannizarro, B. and Croce, C. (1985) Proc. Natl. Acad. Sci. USA 82, 4448–4452.
[7] Rajput, B., Degen, S., Reich, E., Waller, E., Axelrod, J., Eddy, R. and Shows, T. (1985) Science 230, 672–674.
[8] Yang-Feng, T., Opdenakker, G., Volckaert, G. and Francke, U. (1986) Am. J. Hum. Genet. 39, 79–87.
[9] Harris, T. (1987) Prot. Eng. 1, 449–458.
[10] Pohl, G. (1988) Thesis University of Umeå, Umeå University Medical Dissertations, New Series no.207, ISSN 0346-6612.
[11] Weimar, W., Stibbe, J., Van Seyen, A., Billiau, A., De Somer, P. and Collen, D. (1981) Lancet ii, 1018–1020.
[12] Van de Werf, F., Bergmann, S., Fox, K., De Geest, H., Hoyng, C., Sobel, B. and Collen, D. (1984) Circulation 69, 605–606.
[13] Opdenakker, G., Van Damme, J., Bosman, F., Billiau, A. and De Somer, P. (1986) Proc. Soc. Exp. Biol. Med. 182, 248–257.
[14] Einarsson, M., Brandt, J. and Kaplan, J. (1985) Biochim. Biophys. Acta 830, 1–10.
[15] Pohl, G., Kallstrom, M., Bergsdorf, N., Wallen, P. and Jörnvall, H. (1984) Biochemistry 23, 3701–3707.
[16] Levin, E. (1983) Proc. Natl. Acad. Sci. USA 80, 6804–6808.
[17] Wiman, B., Csemicky, G., Marsk, L. and Robbe, H. (1984) Thromb. Haemost. 52, 124–126.
[18] Hoylaerts, M., Lijnen, H. and Collen, D. (1981) Biochim. Biophys. Acta 673, 75–85.
[19] Rijken, D., Wijngaards, G., Zaal-de Jong, M. and Welbergen, J. (1979) Biochim. Biophys. Acta 580, 140–153.
[20] Rijken, D. and Collen, D. (1981) J. Biol. Chem. 256, 7035–7041.
[21] Wallen, P., Pohl, G., Bergsdorf, N., Ranby, M. and Jörnvall, H. (1983) Eur. J. Biochem. 132, 681–686.
[22] Andreasen, P., Nielsen, L., Grøndahl-Hansen, J., Skriver, L., Zeuthen, J., Stephens, R. and Danø, K. (1984) EMBO J. 3, 51–56.
[23] Heussen, C., Joubert, F. and Dowdle, E. (1984) J. Biol. Chem. 259, 11635–11638.
[24] Opdenakker, G., Weening, H., Collen, D., Billiau, A. and De Somer, P. (1982) Eur. J. Biochem. 121, 269–274.
[25] Harris, T., Pattel, T., Marston, F., Little, S., Emtage, G., Opdenakker, G., Volckaert, G., Rombauts, W., Billiau, A. and De Somer, P. (1986) Mol. Biol. Med. 3, 279–292.
[26] Edlund, T., Ny, T., Ranby, M., Hedén, L.-O., Palm, G., Holmgren, E. and Josephson, S. (1983) Proc. Natl. Acad. Sci. USA 80, 349–352.
[27] Browne, M., Tyrell, A., Chapman, C., Carey, J., Glover, D., Grosveld, F., Dodd, I. and Robinson, J. (1985) Gene 33, 279–284.

[28] Kaufman, R., Wasley, L., Spiliotes, A., Gossels, S., Latt, S., Larsen, G. and Kay, R. (1985) Mol. Cell. Biol. 5, 1750–1759.
[29] Fisher, R., Waller, G., Ghrossi, D., Thompson, R., Tizard, R. and Schleuning, W.-D. (1985) J. Biol. Chem. 260, 11223–11230.
[30] Degen, S., Rajput, B. and Reich, E. (1986) J. Biol. Chem. 26, 6972–6985.
[31] Pohl, G., Kaplan, L., Einarsson, M., Wallen, P. and Jörnvall, H. (1984) FEBS Lett. 168, 29–32.
[32] Ashino-Fuse, H., Opdenakker, G., Fuse, A. and Billiau (1984) Proc. Soc. Exp. Biol. Med. 176, 109–118.
[33] Opdenakker, G., Ashino-Fuse, H., Van Damme, J., Billiau, A. and De Somer, P. (1983) Eur. J. Biochem. 131, 481–487.
[34] Opdenakker, G., Billiau, A., Volckaert, G. and De Somer, P. (1985) Biochem. J. 231, 309–313.
[35] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[36] Granelli-Piperno, A. and Reich, E. (1978) J. Exp. Med. 148, 223–234.
[37] Laemmli, U. (1970) Nature 227, 680–685.
[38] Kyhse-Anderson, J. (1984) J. Biochem. Biophys. Methods 10, 203–209.
[39] Tate, K., Higgins, D., Holmes, W., Winkler, M., Heyneker, H. and Vehar, G. (1987) Biochemistry 26, 338–343.
[40] Bohman, D., Bos, T., Admon, A., Nishimura, T., Vogt, P. and Tjian, R. (1987) Science 238, 1386–1392.
[41] Angel, P., Allegretto, E., Okino, S., Hattori, K., Boyle, W., Hunter, T. and Karin, M. (1988) Nature 332, 166–171.