Human endothelial cells contain one type of plasminogen activator

Peter Kristensen*, Lars-Inge Larsson†, Lars S. Nielsen*+, Jan Grøndahl-Hansen*+, Peter A. Andreasen* and Keld Danø*+

*Laboratory of Tumor Biology and †**Unit of Histochemistry, Institute of Pathology, University of Copenhagen, Frederik d. V's Vej 11, 2100 Copenhagen Ø. and +Finsen Laboratory, Finsen Institute, Strandboulevarden 49, 2100 Copenhagen Ø., Denmark

Received 1 January 1984

At least two types of animal plasminogen activating enzymes exist, differing in amino acid sequence, molecular mass and immunological reactivity: the urokinase-type and the tissue-type plasminogen activators. By affinity chromatography with monoclonal antibodies, we have purified the human activators of both types to homogeneity. Using immunocytochemistry with rabbit antibodies raised against these preparations, we now demonstrate that the plasminogen activator present in endothelium of veins and other blood vessels is of the tissue-type. No urokinase-type plasminogen activator immunoreactivity was detected in endothelial cells in the intact organism. These findings support the assumption that mobilization of plasmin for different purposes may involve different types of plasminogen activators, and that the plasminogen activator involved in thrombolysis is of the tissue-type.

Plasminogen activator *Thrombolysis* *Endothelial cell* *Immunocytochemistry* *Proteolytic enzyme*

1. INTRODUCTION

Activation of plasminogen to the trypsin-like protease plasmin is a general mechanism for producing localized extracellular proteolysis [1]. This activation is implicated in a variety of processes, including thrombolysis [2], ovulation [3], embryo implantation [4], the conversion of prohormones [5], cell migration [6], tissue remodelling [7], and tissue degradation in a number of normal as well as pathological conditions [8–12].

Two types of mammalian plasminogen activators, differing in M_r, amino acid sequence and immunological reactivity, have been purified and characterized [10,13–23]. In humans, the urokinase-type (u-PA) has M_r ~52000 and the tissue-type (t-PA) enzyme M_r ~66000. Contributing to the functional diversity of plasmin-mediated proteolysis might be that some processes involve activation of plasminogen by one type of activator while other processes involve activation by the other type. This study was undertaken to elucidate whether one or both types of activators are involved in thrombolysis.

We have raised monoclonal antibodies against both types of plasminogen activators [20,22]. In cultured cells [24] and in a paper model system [25], in which spots of enzyme were stained, using peroxidase-conjugated second antibodies, we found that the monoclonal antibodies were less suited for the staining of fixed enzymes. The monoclonal antibodies were therefore used to produce highly purified enzyme preparations which, in turn, were used to raise polyclonal rabbit antibodies. In the paper model system, the polyclonal antibodies stained pure paraformaldehyde fixed

**Sponsored by the Danish Medical Research Council and the Danish Cancer Society

Abbreviations: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator

Published by Elsevier Science Publishers B V
00145793/84/$3.00

preparations of the respective enzymes with a high affinity, and we now report that endothelial cells of blood vessels in the intact organism contain t-PA but not detectable amounts of u-PA.

2. MATERIALS AND METHODS

t-PA from the culture fluid of Bowes melanoma cells and u-PA from a commercial urokinase preparation (Leo Pharmaceuticals, Copenhagen) were purified to homogeneity, as evaluated by SDS–polyacrylamide gel electrophoresis, by affinity chromatography with monoclonal antibodies [18,22]. Rabbit antibodies were raised by intradermal injection of approx. 10 μg of the respective enzymes inhibited by diisopropylfluorophosphate following the scheme in [15]. The rabbits were bled and the individual sera were compared using immunocytochemical paper models [25]. From the sera selected by this method, IgG was purified by affinity chromatography on protein A–Sepharose. Dilutions for immunocytochemistry were made in TBS-BSA [0.05 M Tris–HCl (pH 7.4) 0.15 M NaCl, 0.25% bovine serum albumin]. Abdominal and breast skin, obtained from patients undergoing plastic surgery, was immediately frozen in isopentane on dry-ice. Cryostat sections were thawed at 4°C in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde, washed in TBS-Triton [0.05 M Tris–HCl (pH 7.4), 0.15 M NaCl with 1% Triton X-100], exposed to normal swine serum and in-

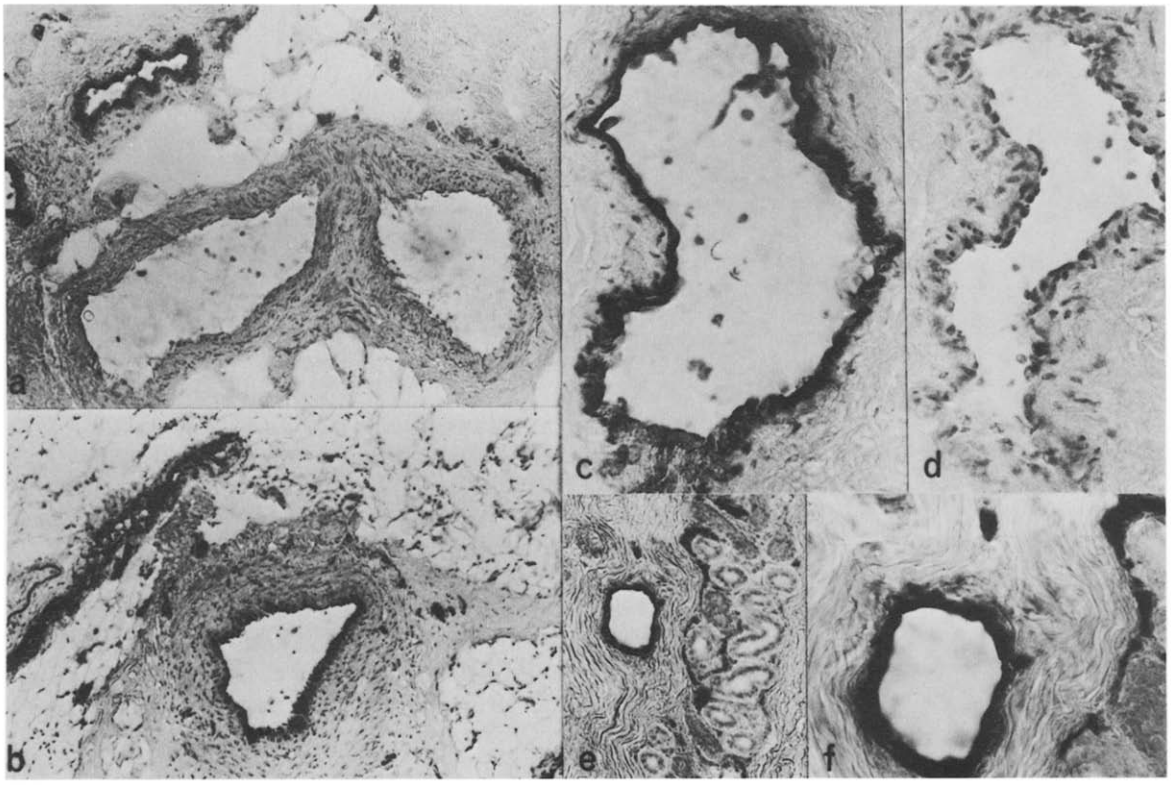

Fig.1. Demonstration of human tissue-type plasminogen activator (t-PA) in endothelial cells of human skin. (a) Micrograph showing the staining of endothelial cells of veins and small vessels, and the lack of staining arteries of human breast skin, with anti-t-PA IgG (1 μg/ml, BSA absorbed); × 104. (b) Staining of endothelial cells of vessels in human abdominal skin with anti-t-PA IgG (1.5 μg/ml); × 82. (c) Staining of endothelial cells of a vein in human abdominal skin with anti-t-PA IgG (1 μg/ml, BSA absorbed); × 203. (d) Staining of section adjacent to (c) with anti-t-PA IgG (1 μg/ml) absorbed with highly purified t-PA (see text); × 203. (e) Staining of vessels in human breast skin with anti-t-PA IgG (1 μg/ml, BSA absorbed); × 104. (f) Enlargement of section (e); × 260.

cubated overnight at 4°C with the primary antibody. The site of antigen–antibody reaction was revealed with the peroxidase–antiperoxidase method in [26], and the peroxidase activity was demonstrated with diaminobenzidine–H_2O_2 [26,27]. Rabbit antiperoxidase–peroxidase complexes and swine anti-rabbit IgG were from Dakopatts, Copenhagen. All washings of sections were performed with TBS-Triton, and sections were lightly counterstained with haematoxylin-eosin or a.m. van Gieson. Control experiments [26,27] included: (i) omission of either the first, second or third layer of antibody; (ii) substitution of anti-t-PA antibody with non-immune rabbit IgG; (iii) absorption of anti-t-PA antibody with highly purified t-PA, bovine serum albumin or urokinase, each coupled to Sepharose. Staining and controls were performed on adjacent sections using the same concentrations of IgG.

3. RESULTS

In the paper model system, the polyclonal antibodies stained pure paraformaldehyde fixed preparations of the respective enzymes with a high affinity, which was approximately the same in the two cases; there was no cross-reaction between the antibodies directed against the two enzymes.

In human skin, the anti-t-PA IgG (1 μg/ml) stained endothelial cells of veins and of some, but not all, smaller arteries (fig.1). The presence or absence of staining of endothelium of the muscular arteries could not be correlated with either the site or the size of the artery. In addition, staining was found in a number of small vessels evenly distributed in the dermis. Counterstaining by van Gieson demonstrated muscle cells surrounding these vessels, which thus could represent small arterioles or precapillary sphincters, but not capillaries.

Staining of human gastric mucosa and lung with anti-t-PA showed a staining distribution in the endothelial cells of vessels similar to that found in the skin (not shown).

Control experiments demonstrated absence of staining after omission of any of the antibody layers, substitution with non-immune IgG or preabsorption of anti-t-PA IgG with purified t-PA. Staining was unchanged after absorption of anti-t-PA IgG with bovine serum albumin and urokinase.

No staining of endothelial cells was observed with anti-u-PA in concentrations up to 50 μg/ml, either in skin, gastric mucosa or lung. As judged from the paper cytochemical models, this concentration of anti-u-PA could detect u-PA if the amount of this enzyme present in the endothelial cells was at least 5% of that of t-PA. Thus, our findings indicate that, of the two types of plasminogen activators, the endothelial cells in the intact organism contain mainly or exclusively t-PA.

4. DISCUSSION

To our knowledge, this report represents the first immunocytochemical study of t-PA. Plasminogen activators are present in very small concentrations, and immunocytochemical studies depend on highly purified enzyme preparations for raising antibodies of sufficient strength and specificity. Affinity chromatography with monoclonal antibodies offers an efficient method to obtain such highly purified enzyme preparations [18,20,22].

The lack of detectable u-PA immunoreactivity in human endothelial cells is in agreement with a recently reported study of the distribution of u-PA immunoreactivity in the mouse [28]. Immunoreactivity was found in several cell types, but no immunoreactivity was observed in any endothelial cells.

Previous investigations of the type of plasminogen activator present in endothelial cells have led to apparently conflicting results. Authors in [29] reported that cultured bovine endothelial cells synthesized both u-PA and t-PA, while those in [30] found that only antibodies to t-PA, and not antibodies to u-PA, inhibited plasminogen activator activity in human endothelium in tissue sections, as measured by the fibrin overlay method described in [31]. The studies in [30], however, did not exclude that the endothelium still contained considerable amounts of u-PA, because the fibrin overlay method preferentially may detect t-PA, as it is known that the activity of t-PA, in contrast to u-PA, is greatly enhanced by fibrin [32,33]. The present study shows that also on a molar basis the endothelial cells in the intact organism mainly contain t-PA and no demonstrable amounts of u-PA. The apparent discrepancy with the results in [29]

may be due to the fact that cultured cells are not necessarily representative of cells in the intact organism with respect to plasminogen activator synthesis.

Our results support the assumption that t-PA, and not u-PA, is involved in thrombolysis, in agreement with reports that in experimental systems t-PA is more effective than u-PA as a thrombolytic agent [34,35]. The findings thus also lend support to the assumption that the mobilization of plasmin for different purposes can involve different types of plasminogen activators.

It is noteworthy that, among human plasminogen activators, mainly u-PA has been used clinically in thrombolytic therapy [36] and that the success of this therapy has only been moderate. It seems likely that t-PA will prove to be more effective for this purpose than u-PA.

ACKNOWLEDGEMENTS

We thank the Department of Plastic Surgery, Rigshospitalet, Copenhagen, for contributing specimens of human skin, and Jette Mandelbaum, Marianne Christensen, Kirsten Lund Jakobsen and Lis Gøricke for excellent technical assistance. This work was supported by the Danish Cancer Society, the Danish Medical Research Council and the Hindsgaul Foundation.

REFERENCES

[1] Reich, E. (1978) in: Molecular Basis of Biological Degradative Processes (Berlin, R.D. et al. eds) pp.155–169, Academic Press, New York.
[2] Collen, D (1980) Thromb. Haemostas. 43, 77–89.
[3] Beers, W.H., Strickland, S. and Reich, E. (1975) Cell 6, 387–394.
[4] Strickland, S., Reich, E. and Sherman, M.I (1976) Cell 9, 231–240.
[5] Virji, M.A.G., Vassalli, J.-D., Estensen, R.D. and Reich, E. (1980) Proc. Natl. Acad. Sci. USA 77, 875–879.
[6] Ossowski, L., Quigley, J.P. and Reich, E. (1975) in: Proteases and Biological Control (Rifkin, D. et al. eds) pp. 901–913, Cold Spring Harbor Laboratory, New York.
[7] Valinsky, J.E., Reich, E. and LeDouarin, N.M (1981) Cell 25, 471–476.
[8] Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rifkin, D.B. and Reich, E. (1973) J. Exp. Med. 137, 85–111.
[9] Vassalli, J.-D., Hamilton, J. and Reich, E. (1976) Cell 8, 271–281.
[10] Danø, K. and Reich, E. (1978) J. Exp. Med. 147, 745–757.
[11] Ossowski, L., Biegel, D. and Reich, E (1979) Cell 16, 929–940.
[12] Unkeless, J.C., Gordon, S. and Reich, E. (1974) J. Exp. Med. 139, 834–850.
[13] Aoki, N. and Von Kaulla, K.N. (1971) J. Lab. Clin. Med. 78, 354–362.
[14] Vetterlein, D., Young, P.L., Bell, T.E. and Roblin, R. (1979) J. Biol. Chem. 257, 7262–7268.
[15] Danø, K., Nielsen, L.S., Møller, V. and Engelhart, M. (1980) Biochim. Biophys. Acta 630, 146–151.
[16] Rijken, D.C., Wijngaards, G. and Welbergen, J. (1981) J. Lab. Clin. Med. 97, 477–486.
[17] Rijken, D.C. and Collen, D. (1981) J. Biol. Chem. 256, 7035–7041.
[18] Nielsen, L.S., Hansen, J.G., Skriver, L., Wilson, E.L., Kaltoft, K., Zeuthen, J. and Danø, K. (1982) Biochemistry 24, 6410–6415.
[19] Steffens, G.J., Gunzler, W.A., Otting, F., Frankus, E. and Flohé, L. (1982) Hoppe-Seylers Z. Physiol. Chem. 363, 1043–1058.
[20] Kaltoft, K., Nielsen, L.S., Zeuthen, J. and Danø, K. (1982) Proc. Natl. Acad. Sci. USA 79, 3720–3723.
[21] Edlund, T., Ny, T., Rånby, M., Heden, L.-O., Palm, G., Holmgren, E. and Josephson, S. (1983) Proc. Natl. Acad. Sci. USA 80, 349–352.
[22] Nielsen, L.S., Hansen, J.G., Andreasen, P.A., Skriver, L., Danø, K. and Zeuthen, J. (1983) EMBO J. 2, 115–119
[23] Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennet, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V. and Collen, D. (1983) Nature 301, 214–221.
[24] Danø, K., Dabelsteen, E., Nielsen, L.S., Kaltoft, K., Wilson, E.L. and Zeuthen, J. (1982) J. Histochem. Cytochem. 30, 1165–1170.
[25] Larsson, L.-I. (1981) J Histochem. Cytochem. 29, 408–410.
[26] Sternberger, L.A. (1979) Immunocytochemistry, John Wiley and Sons, New York.
[27] Larsson, L.-I. (1981) Prog. Histochem. Cytochem. 13, 1–85.
[28] Larsson, L.-I , Skriver, L., Nielsen, L S , Grøndahl-Hansen, J., Kristensen, P and Danø, K. (1984) J. Cell Biol., in press.
[29] Levin, E.G. and Loskutoff, D.J. (1982) J. Cell Biol. 94, 631–636.

[30] Rijken, D.C., Wijngaards, G. and Welbergen, J. (1980) Thromb. Res. 18, 815–830.
[31] Todd, A.S. (1959) J. Pathol. Bacteriol. 78, 281–283.
[32] Wallén, P. (1978) in: Progress in Chemical Fibrinolysis and Thrombolysis (Davidson, J.F. et al. eds) vol.3, pp.167–181, Raven, New York.
[33] Camiolo, S.M., Thorsen, S. and Astrup, T. (1971) Proc. Soc. Exp. Biol. 138, 277–280.
[34] Matsuo, O., Rijken, D.C. and Collen, D. (1981) Nature 291, 590–591.
[35] Mattsson, C., Nyberg-Arrhenius, V. and Wallén, P. (1981) Thrombosis Res. 21, 535–545.
[36] Marder, V.J. (1979) Ann. Int. Med. 90, 802–808.