

Immunohistochemical localisation of tissue plasminogen activator and urokinase in the vessel wall

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SUMMARY Immunoreactive plasminogen activators were studied in tissue sections using a peroxidase method and monospecific antibodies to tissue plasminogen activator produced by a melanoma. Tissue plasminogen activator reactivity was found in skin melanomas and in endothelial and smooth muscle cells of arteries and veins. Vessels of the umbilical cord showed higher reactivity than peripheral vessels. Only faint antiurokinase reactivity was found. By means of the fibrin slide technique, fibrinolytic activity could be shown in peripheral vessel walls but not in the umbilical cord, which suggests that immunoreactivity of tissue plasminogen activator bound to an inhibitor can also be demonstrated. This method may be a useful tool in further studies of tissue plasminogen activator in physiological as well as pathological processes.

Plasminogen is converted into plasmin by two naturally occurring plasminogen activators. The so called tissue plasminogen activator (t-PA) is probably synthesised and released from the vessel wall into the blood stream.¹⁻³ t-PA is also released in cultures of various fetal organs.⁴ Urokinase⁵ is secreted from the kidneys into the urine.⁶ It is secreted in kidney cell cultures^{7,8} and also in cultures of most human fetal organs.⁴

Malignant tumours produce plasminogen activators, and plasminogen activator released from ovarian carcinoma in culture was the first reported to be immunologically identical to urokinase.⁹ Production of urokinase like plasminogen activators has been found in other tumours as well.¹⁰⁻¹⁴ In addition, certain melanoma cell lines produce blood tissue like plasminogen activator.¹⁵ t-PA isolated from malignant melanoma cells had provided sufficient amounts of antigen to raise antibodies against t-PA.⁴

In the present study, using monospecific antibodies against t-PA and urokinase, we examined the histological localisation of immunoreactive plasminogen activators in skin melanoma and in arteries and veins from human umbilical and peripheral vessels.

Material and methods

Umbilical cords were obtained at delivery of normal infants and superficial veins were obtained at surgery. Uterine arteries were obtained at hysterectomy. Specimens were also obtained from patients undergoing operations for treatment of malignant melanoma. The specimens were immediately fixed in phosphate buffered (pH 7.4) formalin (4%) in saline (PBS-formalin) for several hours at room temperature and embedded in paraffin. Sections were cut at 5 μ m, dewaxed by xylene, and hydrated before the immunohistological procedure. Umbilical cord and peripheral vessel specimens were also fresh frozen. Sections 10 μ m thick were taken for examination of fibrinolytic activity according to the method of Todd.¹⁶

ANTIBODIES TO t-PA

t-PA was obtained from the culture medium of Bowe's melanoma cell line (cells provided by Dr D Collen) and purified from the medium according to Wallén *et al.*¹⁷ Antiserum was raised in a goat by two injections (three weeks apart) of 150 μ g of purified activator, emulsified with Freund's complete adjuvant. The injections were given subcutaneously at different sites in the back. The antiserum gave one precipitation line with melanoma conditioned cul-

ture medium in gel diffusion. The melanoma activator showed an immunological reaction identical to that obtained with t-PA purified from human uterine tissue. IgG from the antiserum and normal goat serum was purified by ammonium sulphate precipitation, DEAE-Sephadex chromatography, and Sephadex G-200 gel filtration as described previously.⁴ The IgG completely neutralised plasminogen activator activity in plasma and ordinary euglobulin precipitates. One milligram of IgG completely quenched the activity of 0.07 mg of pure melanoma activator.

ANTIBODIES TO UROKINASE

Urokinase was obtained from Løvens, Copenhagen, and further purified by p-aminobenzamidase-Sephadex chromatography.¹⁸ Low molecular weight (MW 31 000) and high molecular weight (MW 54 000) urokinase were separated by gel filtration.¹⁹ Antiserum against low molecular weight urokinase was raised in a goat as described for t-PA. IgG from the antiserum was prepared in the same way.

IMMUNOHISTOCHEMISTRY

Phosphate buffered saline (PBS, 0.01 M phosphate, pH 7.4) was used for all washings and sera dilutions. All antisera were diluted in PBS plus 0.25% Triton X-100 and 0.25% bovine serum albumin. Antisera to t-PA were used at dilutions of 1/50 to 1/2000 and urokinase antisera at 1/25 to 1/1000. Control sections were incubated with goat non-immune sera or with PBS only. Endogenous peroxidase activity was minimised by treating the sections before incubation with primary antibody with 0.15% H₂O₂ in absolute methanol for 30 min. All sections were rinsed with PBS for 6 h before the first antibody incubation and with PBS plus 0.25% Triton X-100 for 30 min between each subsequent incubation. Sections were incubated with antisera or control sera in a moisture chamber overnight at 4°C. This was followed by incubation with rabbit anti-goat antiserum (1/50, Dakopatts or Nordic) for 30 min. Then, the sections were incubated with the peroxidase-antiperoxidase (PAP) complex (goat PAP, Dakopatts or Nordic, 1/150). Peroxidase activity was demonstrated by incubation of the sections with 3',3'-diaminobenzidine × 4HCl (0.06% DAB/Sigma in 100 ml of 0.05 M Tris buffer, pH 7.6, + 0.01% H₂O₂) for 1 h. Counterstaining was by haematoxylin, and sections were mounted in Eukitt.

Results

MALIGNANT MELANOMA

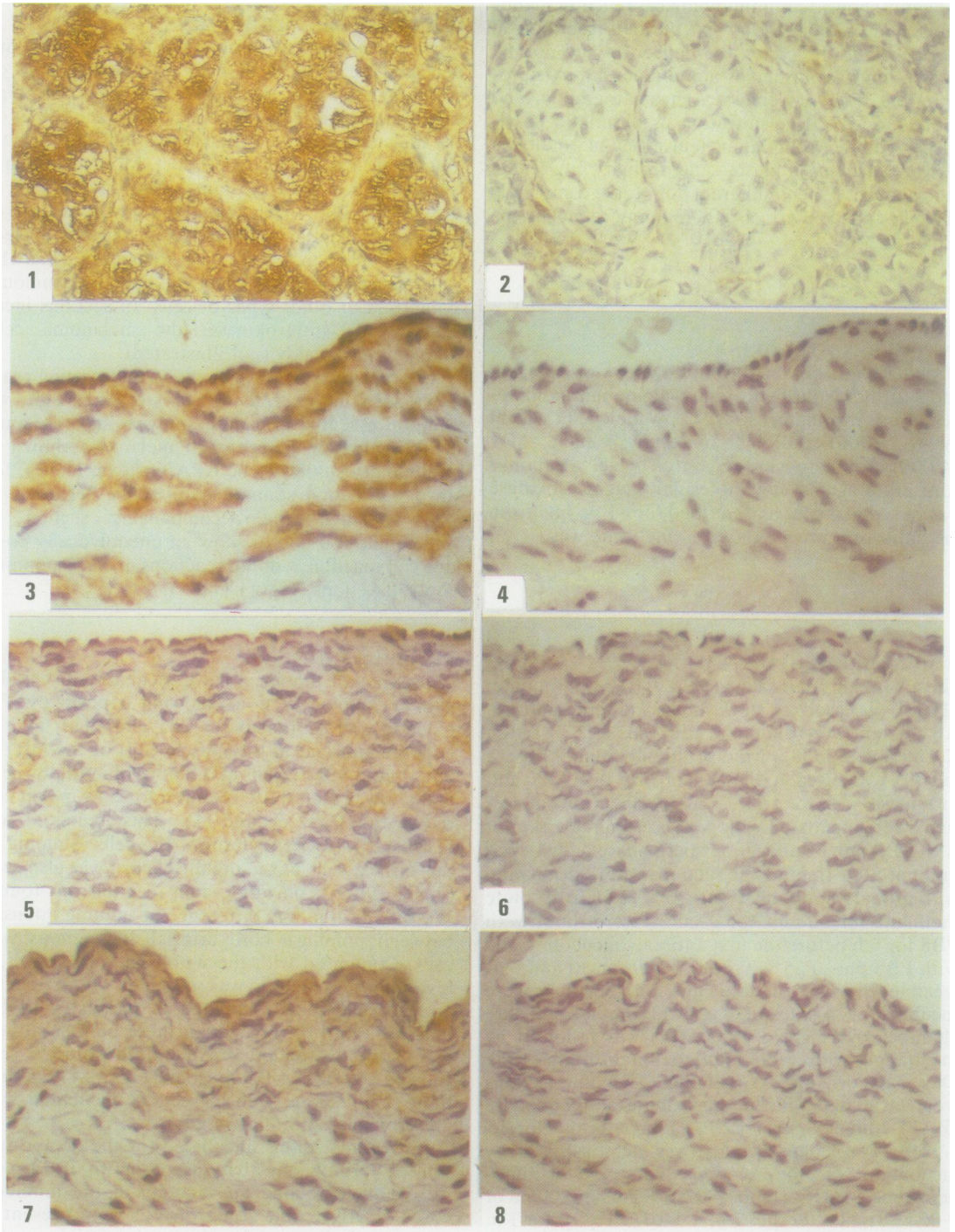
Since our antibody was obtained from a melanoma cell line, we tested a number of different types of melanoma for the presence of t-PA. Heavily pigmented tumours were avoided because the colour of the melanin pigment tended to overlap with the colour of the PAP reaction product. Most melanomas showed a positive t-PA reaction at dilutions up to 1/500 to 1/1000. In particular, tumours composed of large, clear epithelioid type cells showed a clear positive reaction in the cell cytoplasm (Fig. 1), whereas variable results were obtained with tumours composed of other types of cells. In sections incubated with antiurokinase, the melanoma cells stained only weakly (not illustrated).

In order to reduce problems of interpreting reactions seen in other tissues (vessel walls etc), we found it essential to include in every separate anti-t-PA incubation a section of melanoma shown in preliminary studies to be t-PA positive. Hence, for an experimental stain reaction to be regarded as t-PA positive, the accompanying section of melanoma also had to show an unequivocally positive cell reaction, with no reactions in corresponding control sections (Figs. 1 and 2).

ARTERIES AND VEINS

In sections of umbilical cord, incubated in parallel with melanoma sections showing a positive t-PA reaction, t-PA reactivity was recorded in the endothelium and in the muscle cells of the arterial and venous vessel walls at dilutions of antibody generally lower than those required to show melanoma reactivity (Figs. 3 and 4). This reaction was restricted to the cytoplasm of the cells. Staining of the surface cells of the umbilical cord was also seen (cf Fig. 9), but no other stain reaction was found. We did not find any difference in staining intensity between umbilical cord arterial and venous wall cells. Compared with the umbilical cord vessels, a less intense reaction was obtained in peripheral arteries and veins incubated in parallel with the umbilical cord (cf Fig. 9). In these peripheral vessels, both the endothelium and the muscle cells showed a positive t-PA reaction (Figs. 5–8) but only faint staining was seen in sections incubated with antiurokinase (not illustrated).

In frozen sections, incubated for the demonstration of fibrinolytic activity according to Todd,¹⁶ we were unable to find any activity in the umbilical cord. Clearly visible zones of lysis were present in sections of the peripheral vessels, mainly in the adventitia of the vessel walls.



Figs. 1 and 2 Portion of a malignant melanoma of skin, composed of large, clear epithelioid type of cells. Section in Fig. 1 was incubated with anti-t-PA 1/200. Fig. 2 is the control section incubated with non-immune serum. A brown reaction product indicating the presence of t-PA is clearly seen within the cytoplasm of the tumour cells. A brown staining in control sections corresponds to melanin pigment. $\times 400$.

Figs. 3 and 4 Portion of umbilical cord artery, incubated with anti-t-PA 1/80 (Fig. 3). Control section Fig. 4 (see also Fig. 9). Clearly positive staining is found in the endothelium and in the muscle cells. A similar degree of staining was seen in the umbilical cord vein endothelium and muscle cells. $\times 200$.

Figs. 5 and 6 Portion of peripheral artery, incubated with anti-t-PA 1/80 (Fig. 5). Control section Fig. 6 (see also Fig. 9). Positive staining is seen in the endothelium and in the muscle cells, the intensity of staining being less than in corresponding umbilical cord arteries (cf Fig. 3). $\times 200$.

Figs. 7 and 8 Portion of peripheral vein, incubated with anti-t-PA 1/80 (Fig. 7). Control section Fig. 8 (see also Fig. 9). Positive staining is found in the endothelium and in the muscle cells, the intensity being less than in corresponding umbilical cord vein (cf Fig. 3). $\times 200$.

Discussion

We are unaware of any previous report showing the tissue localisation of immunoreactive plasminogen activator. The published work indicates that proliferating cells, being tumour or embryonal, tend to be appreciable producers of plasminogen activator.^{20-25,94} In tumours, it has been suggested that the metastatic potential may be related to type IV collagenase activity, due to plasmin activation of the enzyme.^{26,27} Our findings are in accordance with the concept of proliferating cell t-PA production. Thus we found that higher dilutions of antibody, indicating greater amounts of t-PA in the cells, could be used to demonstrate melanoma immunoreactivity rather than to demonstrate blood vessel wall reactivity. Also, we found that embryonal cord vessel immunoreactivity was higher than corresponding peripheral blood vessel reactivity. Interestingly, the t-PA immunoreactivity in umbilical cord vessel walls was not associated with any fibrinolytic activity using the Todd technique. In contrast, fibrinolysis was clearly seen in the walls of the peripheral blood vessels. Our findings indicate that the embryonal vessel t-PA is present in a latent form, presumably as an enzyme-inhibitor complex.²⁸

To check the validity of the method and the t-PA antibodies, sections of malignant melanomas were used and the presence of t-PA could be clearly identified (Fig. 1). The results also show that in veins as well as in the arteries t-PA is found not only in the endothelium but also in the muscle wall. In several studies using the histochemical method of Todd¹⁶ as modified by Pandolfi¹ fibrinolytic activity of veins was mainly restricted to the adventitia.^{29,30,1} Absence of endothelial fibrinolytic activity in these studies and the presence of immunoreactive t-PA in the present study may be explained by recent observations that an inhibitor of t-PA is present and released from endothelial cells.^{2,31,28}

We could not completely exclude the presence of urokinase in the vessel walls. Using the histochemical technique, Ljungnér *et al*³² found that the activity of t-PA was completely quenched by antibodies against t-PA but unchanged when antibodies against urokinase were mixed in the fibrin film. It is possible that some enzymatically inactive preurokinase³³⁻³⁶

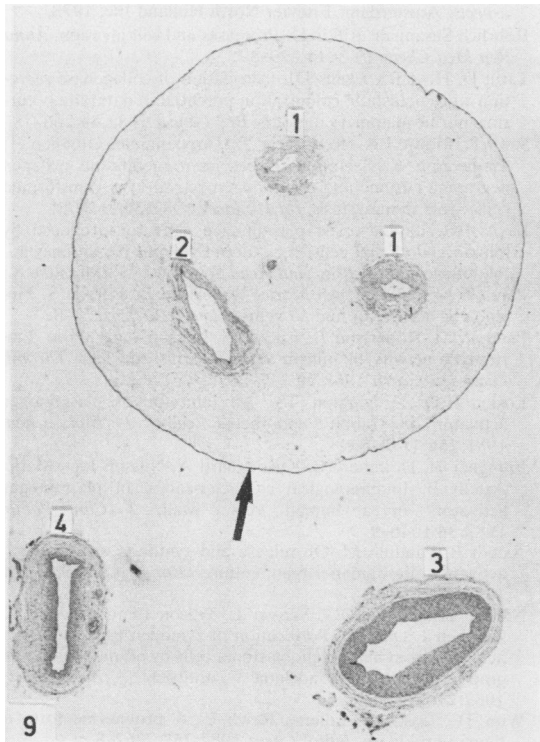


Fig. 9 Cross section of umbilical cord, with the two arteries (1) and the vein (2), mounted in the same block as a separate peripheral artery (3) and vein (4). In such sections, t-PA reactivity has been recorded in the surface cell layer (arrow), in endothelial cells, and muscle cells of the vessel walls, with umbilical cord vessels showing a higher degree of activity than peripheral vessels (cf Figs. 3-8). $\times 20$.

which possesses immunoreactive properties could be present in the vessel wall. Holmberg *et al*⁴, however, found secretion into the medium of t-PA as well as of urokinase in organ culture of various fetal organs, but aorta explants released only t-PA. In cultured human endothelial cells, Levin²⁸ did not find any evidence for secretion of urokinase like plasminogen activator.

The method and the results presented here might prove useful for selection of cell structures for culture and production of t-PA and also for experimental studies of t-PA activity in physiological and pathological processes.

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