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COMPONENTS OF THE PLASMINOGEN ACTIVATION SYSTEM IN UVEAL MELANOMA—A CLINICO–PATHOLOGICAL STUDY

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SUMMARY

In tumour development, proteases such as plasminogen activators (PAs) play a role in degradation of the extracellular matrix and other tissue barriers. Recently, we demonstrated that plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of cutaneous melanocytic tumour progression. In this study we investigated the expression and distribution of the various components of the PA system and the presence of PA enzyme activity in 45 freshly frozen primary uveal melanomas with known follow-up (14 spindle and 31 non-spindle type) and in metastases ($n=5$). Tissue-type PA (t-PA) was found in endothelium of blood vessels and in tumour cells in almost all lesions, and was markedly present at the invasive front (towards the sclera and Bruch's membrane), but no correlation with tumour-related death could be established. Urokinase PA (u-PA) was expressed focally, by only five non-spindle cell melanomas but in all metastases. u-PA expression correlated with occurrence of metastasis. u-PA receptor (u-PAR) was present in one-third of all the tumours examined. Plasminogen activator inhibitors (PAI-1 and PAI-2) were found only focally in approximately 10 per cent of the lesions. Staining of t-PA, u-PA, and PAI was observed in all the metastases. We conclude that in uveal melanoma, u-PA expression may be associated with metastatic disease and accordingly with a poor prognosis. Further research on a larger group of tumours with known follow-up is needed to establish whether u-PA positivity is of additional prognostic value in uveal melanoma.

KEY WORDS—Uveal melanoma, plasminogen activation, proteolysis, immunohistochemistry, urokinase.

INTRODUCTION

To migrate from the primary tumour into blood vessels and to home and grow at a distant site in the body, tumour cells need an extensive repertoire of proteins.[1] For migration through and break-down of tissue barriers, proteases are required.[2] An important proteolytic system involved in tumour progression comprises the proteins of the plasminogen activator system.[3,4] The two known plasminogen activators (PAs) are tissue-type (t-PA) and urokinase-type plasminogen activator (u-PA). PA activity can be inhibited by specific PA inhibitors, PAI-1 and PAI-2.[5] u-PA activity can be focused at the cell surface by binding to its receptor (u-PAR). Also, u-PA activity can be enhanced by binding to the receptor, as the conversion of the inactive pro-u-PA to active u-PA is facilitated on the cell surface.[6,7] Furthermore, by rapid internalization of u-PAR saturated with u-PA:PAI-1 and recycling of u-PAR, the availability of u-PAR for u-PA binding is

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ensured.[8–10] The saturation of the u-PAR is either autocrine or paracrine.[11–13]

The actual involvement of PAs in tumour cell migration and metastasis formation has been shown in several model systems.[14–16] Elevated levels of u-PA, u-PAR, and PAI in human malignant tumour tissue relative to the benign precursor lesion or normal tissue have been reported for tumours of diverse origin.[17–22] Furthermore, u-PA content has been shown to be a valuable prognostic marker for mammary carcinoma.[19,23] The role of t-PA in tumour progression, however, is more ambiguous. Lower levels of t-PA in tumours compared to benign lesions have been reported for tumours of the mammary gland[24] and the ovary.[21] Regarding cutaneous melanoma, some reports indicate a high expression of t-PA in melanoma cell lines[16,25,26] and cutaneous melanoma lesions.[25,26,39]

We recently demonstrated, both in a nude mouse model[16] and in fresh human cutaneous melanocytic lesions,[22] a possible role for the PA system in melanoma tumour progression. u-PA and PAI expression was related to a high metastatic potential of melanoma cell lines in nude mice.[16] This was reflected in the *in vivo* situation in fresh human melanocytic lesions: u-PA, PAI-1 and PAI-2, but also t-PA and u-PAR emerge in late stages of cutaneous melanocytic tumour progression.[22]

In this study, we extended our investigation on the involvement of the PA system in melanoma tumour progression to melanoma of the uvea. Melanoma of the uvea is the most common primary intraocular malignancy in adults[27] and several factors have been found to influence the prognosis. These include tumour cell type (spindle cell type has a better prognosis than non-spindle cell type) and tumour diameter. Uveal melanomas differ from cutaneous melanomas in several aspects. Uveal melanomas spread haematogenously, preferentially to the liver, whereas cutaneous melanomas primarily metastasize by lymphatics. The estimated 5-year survival rate for uveal melanoma is 75 per cent[28] and is comparable to that for cutaneous melanoma.[29] The aim of this study was to investigate the expression of the different components of the PA system in uveal melanoma. The presence of these proteins was studied on fresh human lesions of primary uveal melanoma and metastases of uveal melanoma using immunohistochemistry and *in situ* zymography.

MATERIALS AND METHODS

Tissue specimens

Specimens from primary uveal melanomas were obtained from 45 patients treated at the Department of Ophthalmology, Erasmus University, Rotterdam, The Netherlands between 1987 and 1992. The enucleated eyes were transported on melting ice to the Department of Pathology. After transillumination, the eyes were cross-sectioned through the tumour and part of the tumour was snap-frozen in OCT compound (Tissue-tek) and stored at $-70°C$. The remainder of the eye was fixed in formalin and embedded in paraffin.

Five uveal melanoma metastases were obtained from three patients with skin ($n=2$), heart ($n=2$), and liver ($n=1$) metastases. From four metastases, no primary tumour was available. From one metastasis, the primary tumour was also included in this study. Metastatic tumours were obtained from the Departments of Pathology of Nijmegen ($n=4$) and Rotterdam ($n=1$).

Antibodies

Rabbit anti-human t-PA and u-PA polyclonal antibodies were from the Gaubius Institute, Leiden, The Netherlands and have been used in earlier studies.[16,22] Monoclonal antibodies against human u-PAR (# 3936) and human PAI-1 (# 380) were purchased from American Diagnostica Inc., Greenwich CT, USA. The rabbit and goat polyclonal antibodies against human PAI-2 were a generous gift from E. Schüler, Behring Werke AG, Marburg, Germany. All antibodies against components of the PA system were used in a previous study.[22] In that study, using parallel sections for mRNA *in situ* hybridization, we established with these antibodies that cells producing the mRNA also contained the protein. Detection of the melanocytic differentiation marker gp-100 with monoclonal antibody NKI-beteb[30] was used to establish the melanocytic origin of the tumours.

Immunohistochemistry

For immunohistochemistry, 4 μm cryostat sections were air-dried overnight at room temperature and stored at $-80°C$ until use. Dilutions of the antibodies and staining procedure were as used before.[22] Stainings with monoclonal antibodies were developed with an ABC technique, and those with polyclonal antibodies with peroxidase

labelled anti-rabbit or anti-goat secondary antibody. Bound antibodies were visualized by using 3-amino-9-ethylcarbazole as a substrate for peroxidase. After counterstaining the nuclei with Papanicolaou's Harris solution, sections were mounted with Kaisers glycerin (Merck, Darmstadt, Germany). A lymph node metastasis from a cutaneous melanoma, previously found to express abundant u-PA, u-PAR and PAI-1 protein, was used as a positive control; for t-PA, the staining of blood vessels served as an internal control, and for PAI-2, staining of sections from fresh human placenta served as a positive control. An incubation in which the first antibody was omitted served as a negative control.

Score

For each section, the percentage of positively labelled melanocytic cells was estimated. Each section was assigned to one of the following categories: 0 per cent, 1–5 per cent, 5–25 per cent and 25–100 per cent positivity. Notes were taken of other staining components (fibroblast-like cells, extracellular matrix) among the melanocytic areas. Sections were scored independently by two observers (CMM, MRvB). Discrepancies were found in only a few cases. These cases were re-evaluated jointly until agreement was reached.

In situ *zymography*

The technique has been described in great detail by Sappino *et al.*[31] and De Vries *et al.*[22] Briefly, 8 μm cryostat sections were covered with an overlay mixture containing dry milk, plasminogen, and agar. At spots where plasminogen activation occurs, lysis of the gel takes place, making the gel transluminant. Addition to the gel of polyclonal antibodies against t-PA or u-PA allows discrimination between the two plasminogen activators. In addition, amiloride also inhibits u-PA specifically. With each incubation session, xenograft lesions of the human melanoma cell lines BLM or MV3, known to express abundant u-PA and hardly any t-PA,[16] were included as a positive control.

RESULTS

Clinico-pathological data

A histopathological diagnosis was obtained on paraffin-embedded tissues. The cell type of the tumours was classified as follows, using the presence or absence of any epithelioid cells as the criterion: spindle ($n=14$, tumours containing only spindle cells) or non-spindle ($n=31$, a combination of mixed and purely epithelioid tumours).[32] The other variables that we measured included greatest tumour diameter, tumour height, the presence of episcleral growth, and the presence of vascular invasion. All but two specimens examined contained more than 50 per cent viable tumour cells.

Follow-up was available in all patients included in this study; from one of these patients, a freshly frozen subcutaneous mestatatic lesion was obtained. Eight out of 45 patients died from tumour-related death (TRD); three died of other causes. The total mean follow-up was 34·7 months.

In ten eyes, extrascleral growth was noted; three melanomas showed obvious vascular invasion.

Immunohistochemistry

All primary tumours and metastases stained with NKI-beteb,[30] recognizing gp-100, a melanocytic differentiation marker (not shown).

Tissue sections were stained for t-PA, u-PA, u-PAR, PAI-1 and PAI-2. Typical examples of immunohistochemical staining are shown in Fig. 1. The total number of lesions which stained for the different components is summarized in Table I. In Fig. 2, the percentage of stained melanocytic cells per cell type of uveal melanoma is displayed.

t-PA

In all lesions, abundant t-PA immunoreactivity was found in the wall of blood vessels (Fig. 1a). The correlation between t-PA expression and cell type is shown in Fig. 2: 42 out of 45 tumours contained t-PA; however, a moderate to abundant staining (>5 per cent tumour cells, $n=16$) was seen predominantly for non-spindle cell type tumours (14/16). Interestingly, in four tumours t-PA staining was strikingly in nests of pleomorphic epithelioid cells. These cells were located around the vessels at both invasive fronts, i.e., towards the sclera (Fig. 1b) and towards Bruch's membrane (Fig. 1c). There was no relationship between the percentage of t-PA positivity and TRD. All metastases contained t-PA.

u-PA and u-PAR

While no u-PA expression could be detected in any of the spindle cell-type tumours tested, focal


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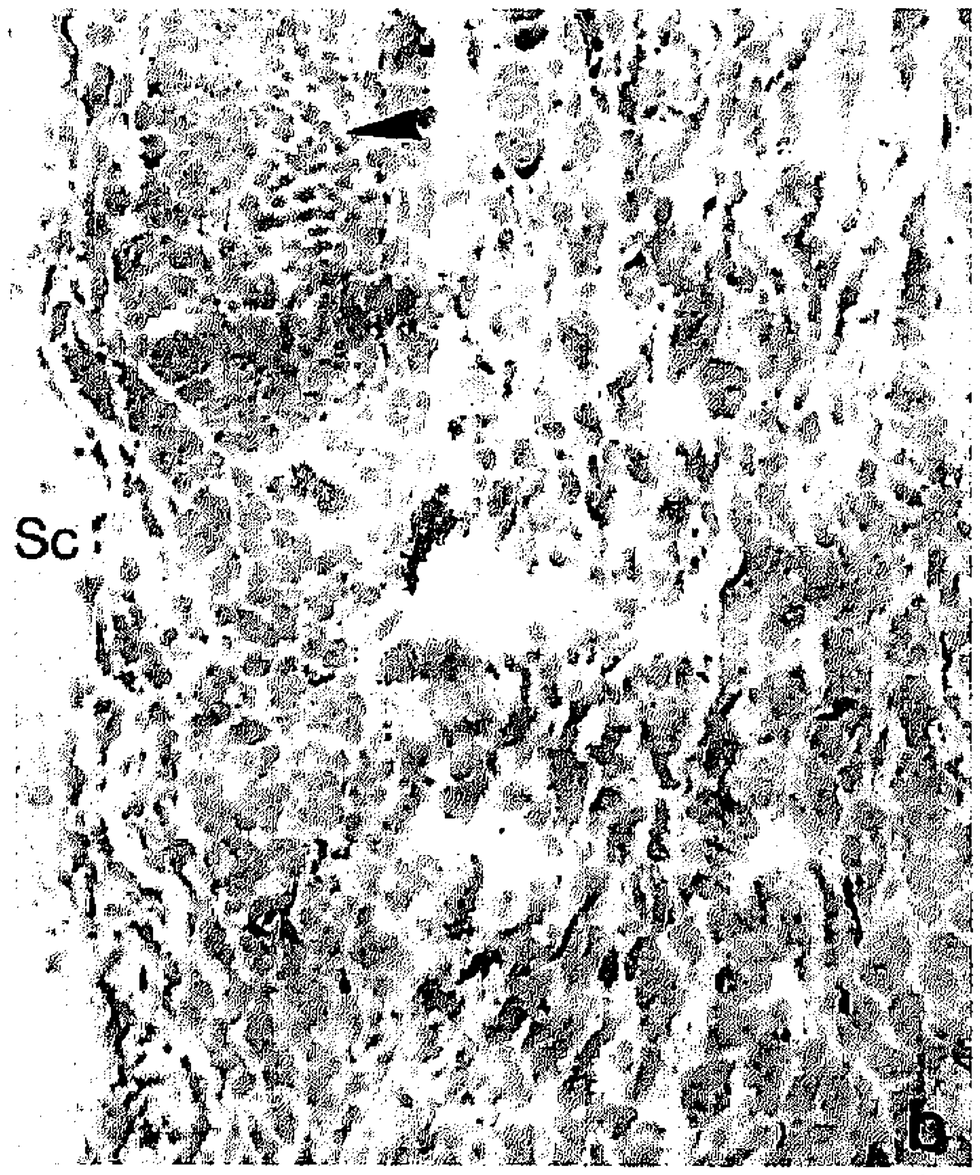
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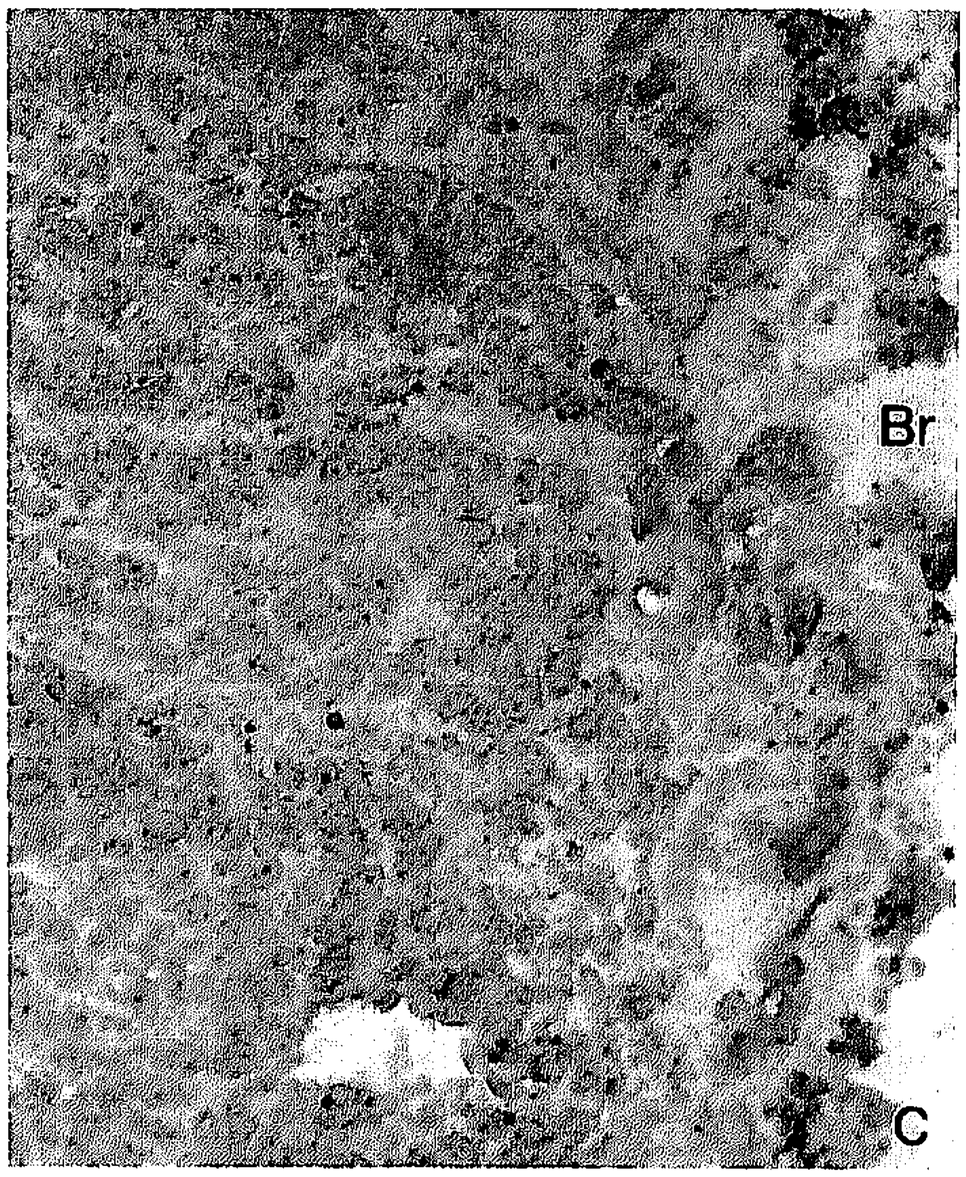
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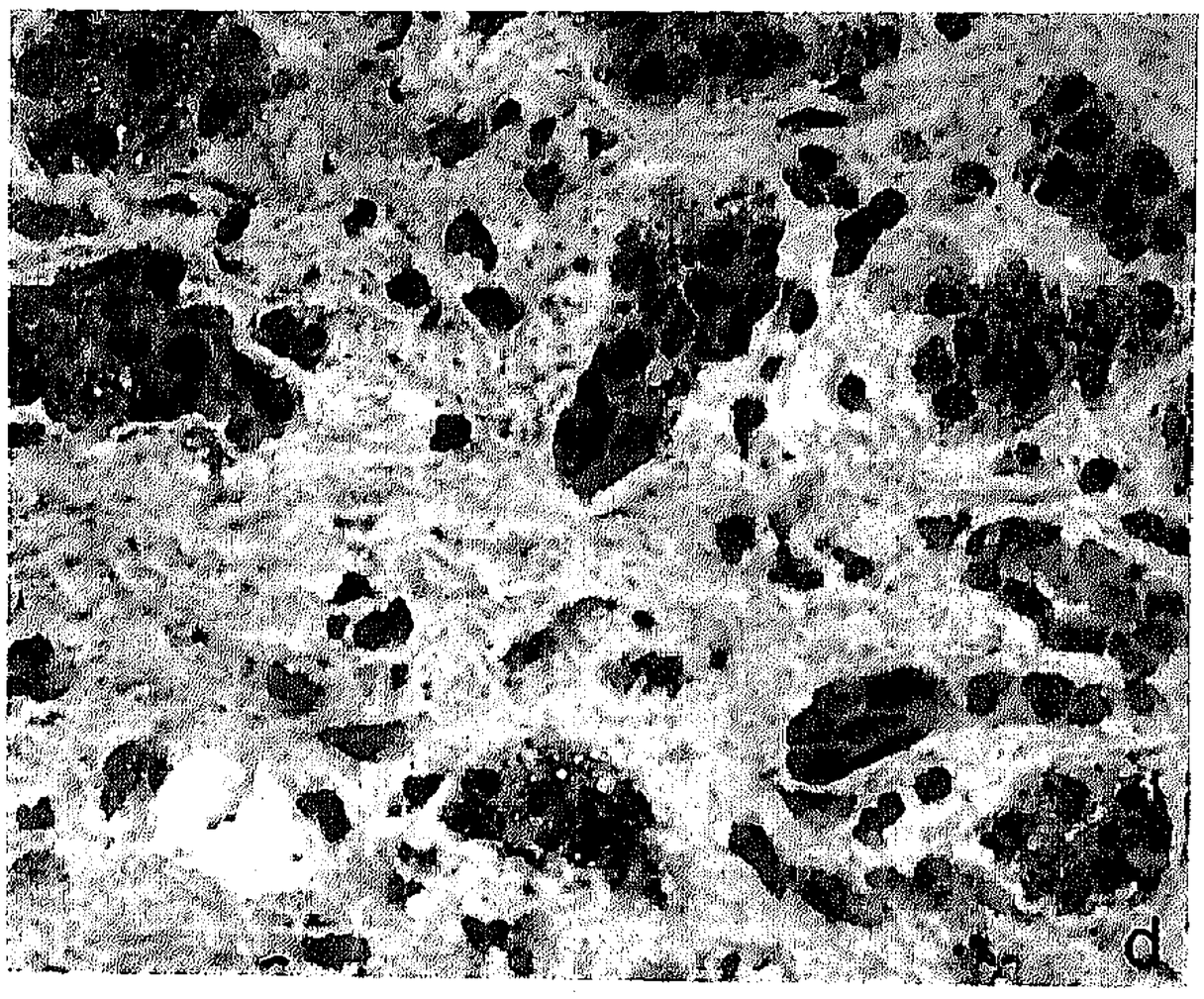
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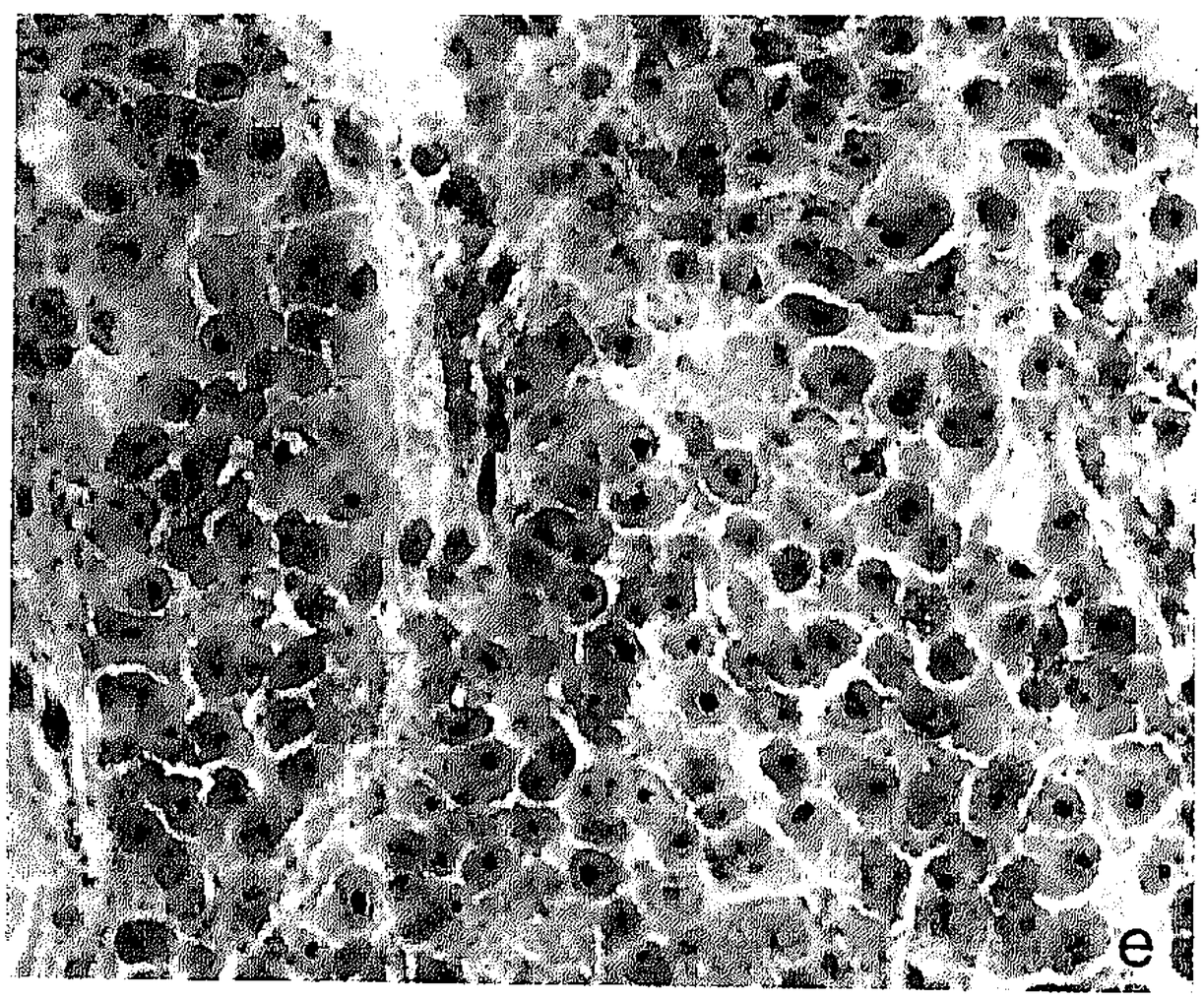
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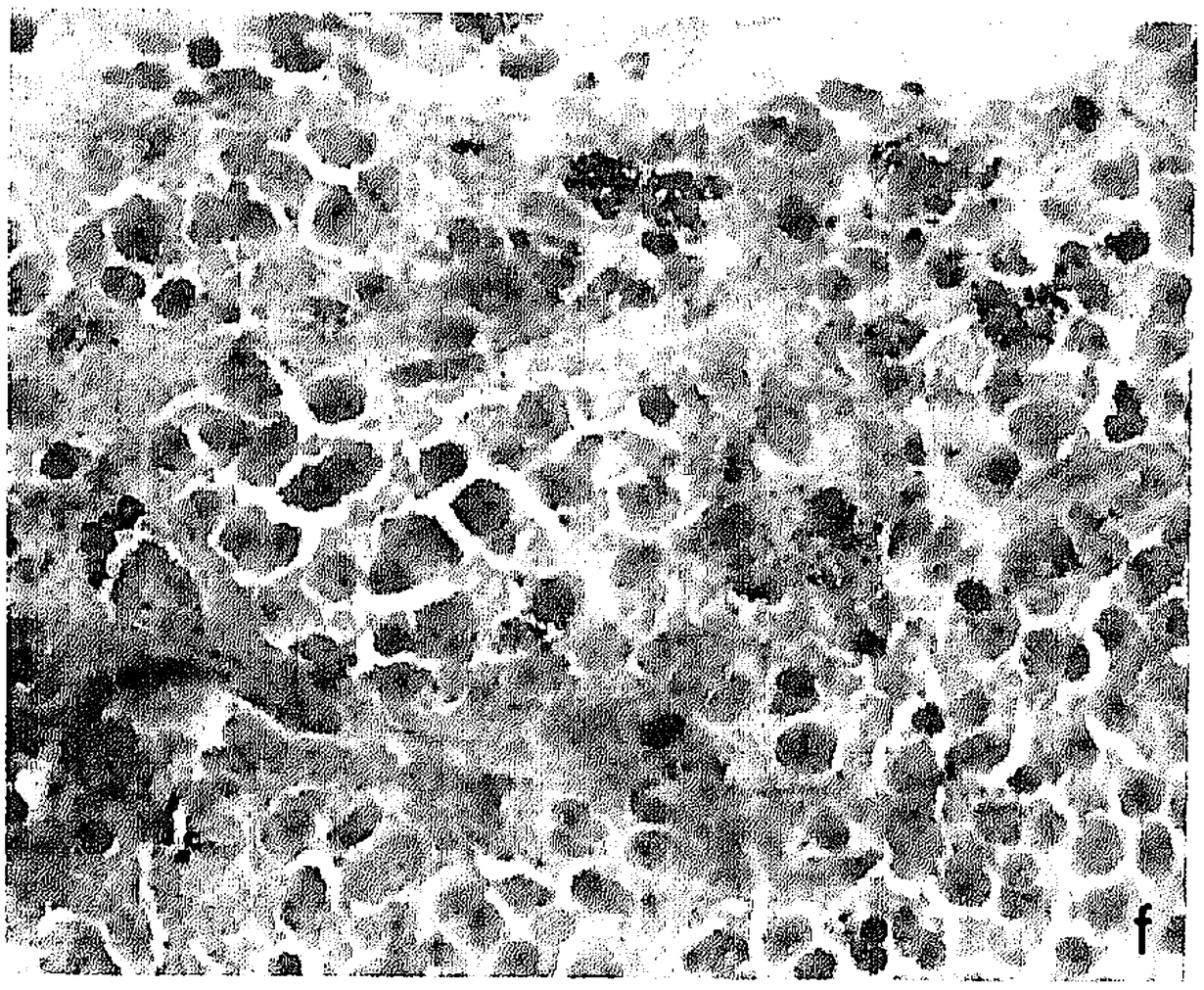
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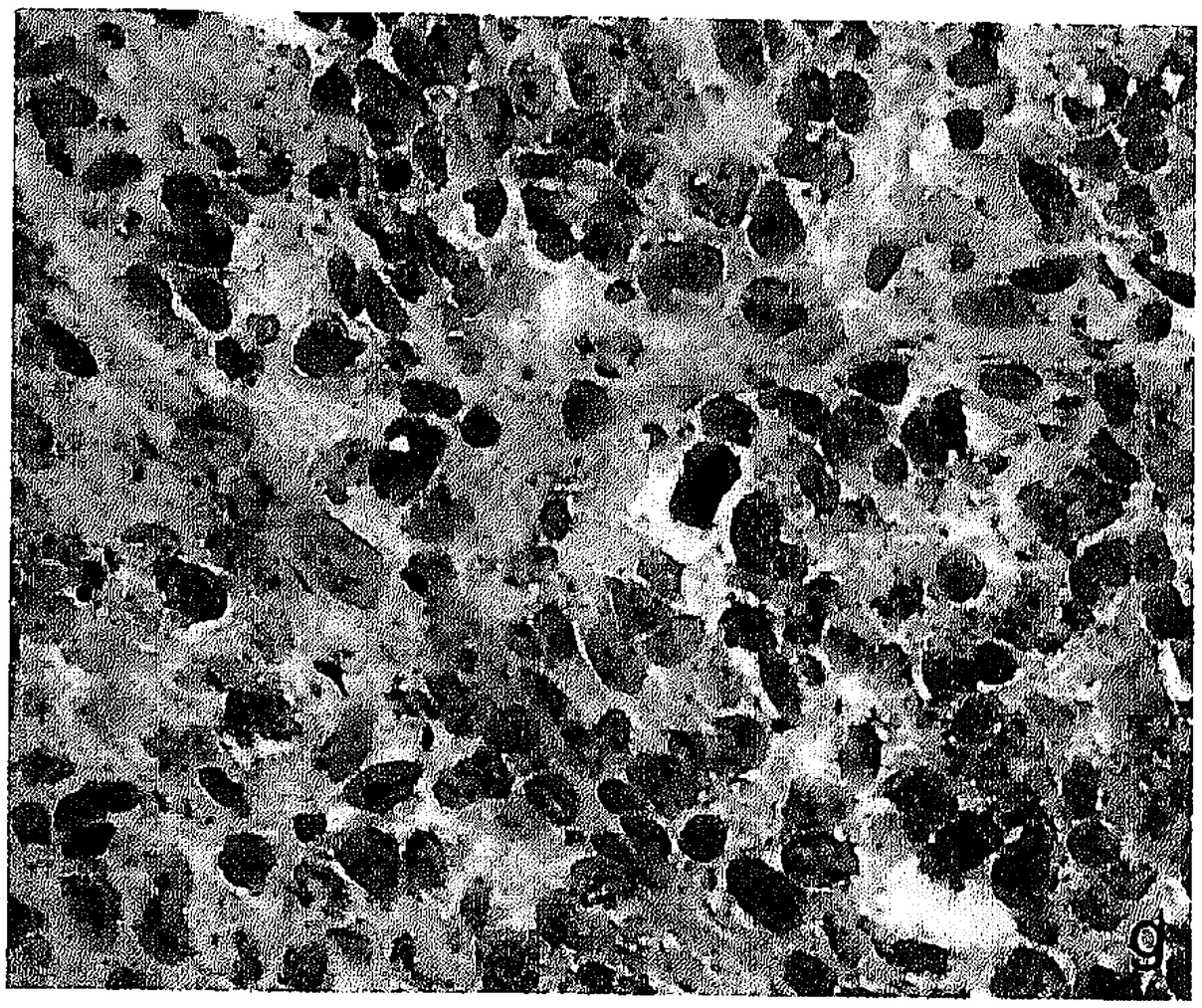
g

u-PA staining (<5 per cent) of tumour cells as well as staining of fibroblast-like cells was detected in five non-spindle cell tumours. In two cases, u-PA expression correlated with TRD; one patient was known to have metastases, and for two patients follow-up was short (32 and 18 months). All five metastases expressed u-PA protein (Fig. 1d).

Focal u-PAR immunoreactivity (<5 per cent) was seen in 15 tumours; 11 out of 15 were non-spindle cell tumours. Both tumour cells (Fig. 1e) and stromal cells showed a cytoplasmic staining aspect. Two metastases contained u-PAR.

PAI-1 and PAI-2

PAI-1 protein was observed in only six primary tumours (three were of the spindle cell type), but in the majority of metastatic tumours (four out of five). The localization was in tumour cells and in the extracellular matrix (Fig. 1f).

PAI-2 protein was observed in 5 out of 45 tumours and was found only in lesions of the non-spindle cell type. PAI-2 was expressed both by tumour cells and by fibroblast-like cells (Fig. 1g). One metastasis contained PAI-2.

In two out of five u-PA-positive tumours, u-PAR and PAI were found in the same lesion, both patients died from metastatic disease. One of these two patients showed expression of all five components. A trend towards higher expression (higher percentage of immunoreactive cells) and a higher percentage of positive lesions in non-spindle compared to spindle cell lesions for t-PA, u-PA, u-PAR, and PAI-2 can be deduced from Fig. 2.

In situ *zymography*

In situ zymography was performed on sections of all lesions studied. In all cases, t-PA activity was found, often in a dotted pattern (Fig. 3, first row). By comparing the lysis pattern with the haematoxylin and eosin-stained sections, we could assign t-PA activity to blood vessels. Lysis in tumours with t-PA immunoreactivity in the highest category (25–100 per cent) occurred faster and was more generalized throughout the section (compare Fig. 3, first and second row). Also in a few metastases we could detect t-PA activity in an area with mainly tumour cells (Fig. 3, third row). Except for the positive control BLM and MV3 xenografts (Figure 3, fourth row), u-PA activity could not be detected in any of the tumours studied.

Table I—Immunohistochemical staining of the components of the plasminogen activation system in primary uveal melanomas and in metastases of uveal melanoma

	t*	s	e	Total
Primary uveal melanoma (n=45)				
Spindle (*n*=14)				
t-PA	13	3	3	13
u-PA	0	0	0	0
u-PAR	3	1	0	3
PAI-1	2	2	1	3
PAI-2	0	0	0	0
Non-spindle (*n*=31)				
t-PA	29	16	5	29
u-PA	5	2	0	5
u-PAR	12	5	0	14
PAI-1	1	3	2	3
PAI-2	5	3	1	5
Metastases of uveal melanoma (n=5)				
t-PA	4	1	3	5
u-PA	5	4	1	5
u-PAR	2	0	0	2
PAI-1	3	1	1	4
PAI-2	0	1	0	1

*t=Staining of tumour cells; s=stromal cells; e=extracellular matrix stained in these lesions.

DISCUSSION

We recently studied the involvement of plasminogen activation in melanoma tumour progression, both in a nude mouse system and in the *in vivo* situation on sections of fresh human

Fig. 1—Immunohistochemical staining of components of the plasminogen activator system in fresh human uveal melanoma lesions. Typical staining examples in primary lesions (a–c, e–g) and a metastasis (d) are shown. (a) t-PA protein in blood vessels. (b) t-PA present in tumour cells at the scleral (Sc) invasive front (arrow-head shows a nerve). t-PA present in tumour cells adjacent to the retinal pigment epithelium where Bruch's membrane (Br) is ruptured (rupture not seen). (d) u-PA is present in tumour cells in this metastasic lesion. (e) u-PAR is located at the invasive front towards the sclera in this lesion. (f) Network like PAI-1 distribution in the extracellular matrix surrounding tumour cells. (g) PAI-2 was found focally in the stroma and in tumour cells (not shown). Cell types: spindle cell tumours (a) and non-spindle cell tumours (b–c, e–g)

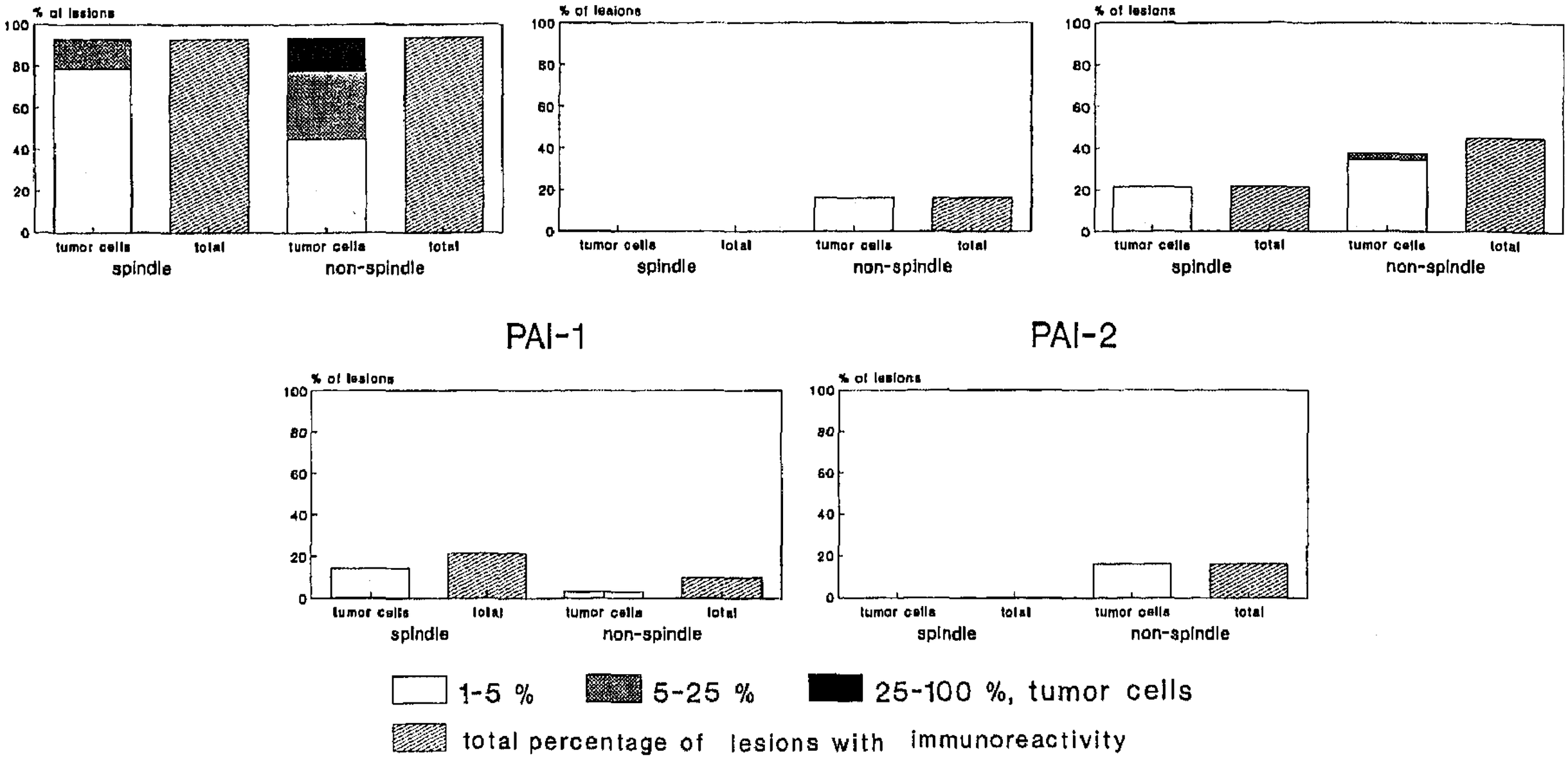

Fig. 2—Percentage of immunohistochemically stained tumour cells and percentage of immunoreactive lesions including staining of extracellular matrix and stroma cells (e and s in Table I), expressed as a percentage of the total number of lesions. Fourteen tumours had purely spindle cell type (spindle) morphology and 31 tumours (either of mixed origin or purely epithelioid) contained epithelioid cells (non-spindle). In non-spindle cell melanomas, a higher percentage of positive lesions and higher percentages of positive cells were observed for t-PA, u-PA, u-PAR, and PAI-2. Metastases are excluded from this diagram due to the low numbers included in this study. See also Table I

cutaneous melanocytic lesions. u-PA, PAI-1, and PAI-2 expression in human melanoma cell lines correlated with a high metastatic capacity in nude mice. All cell lines contained t-PA and u-PAR.[16] In human cutaneous melanocytic lesions, u-PA, PAI-1, and PAI-2 but also t-PA and u-PAR, were found in the most malignant lesions only (advanced primary melanomas and melanoma metastases).[22] Here, we extended our study on the involvement of the plasminogen activation system in melanocytic lesions to uveal melanoma. Once the diagnosis of metastatic disease in uveal melanoma is made (usually by fine needle aspiration), the subsequent median survival is only 2–4 months.[33,34] Therefore metastatic tissue is hard to access and only five metastases were available.

Little is known concerning the presence of proteases in uveal melanoma lesions. The involvement of proteases in metastatic spread of uveal melanoma has recently been suggested by Cottam *et al.*,[35] who detected the 72 and 92 kD type IV collagenase in the culture medium of 15 primary cultures of uveal melanoma. Furthermore, t-PA activity in supernatants of primary cultures of uveal melanoma seemed to correlate with scleral invasion in the tumour lesion, whereas no u-PA activity could be detected.[36] Interestingly, the choroid itself expresses t-PA[37] and u-PA.[38]

In our study, t-PA protein was expressed by tumour cells in the vast majority of the lesions. Expression of t-PA in uveal melanoma therefore does not differ from previous reports on melanoma as a source for t-PA: abundant t-PA was found in melanoma cell lines[16,26,27] and in cutaneous melanocytic lesions.[26] t-PA was expressed in a larger percentage of the tumour cells in non-spindle cell tumours. Immunoreactive cells often had a perivascular localization. The localization of t-PA-positive cells at both the scleral and Bruch's membrane invasion front was remarkable. This observation is in agreement with the work of Cottam *et al.*,[36] who found that elevated t-PA activity in primary cultures of uveal melanoma correlated with scleral invasion. t-PA positivity was noted in varying percentage (0 per cent up to 25–100 per cent categories) in patients who had died from TRD as well as in patients which were still alive. No relationship between the extent of t-PA positivity and TRD could be established.

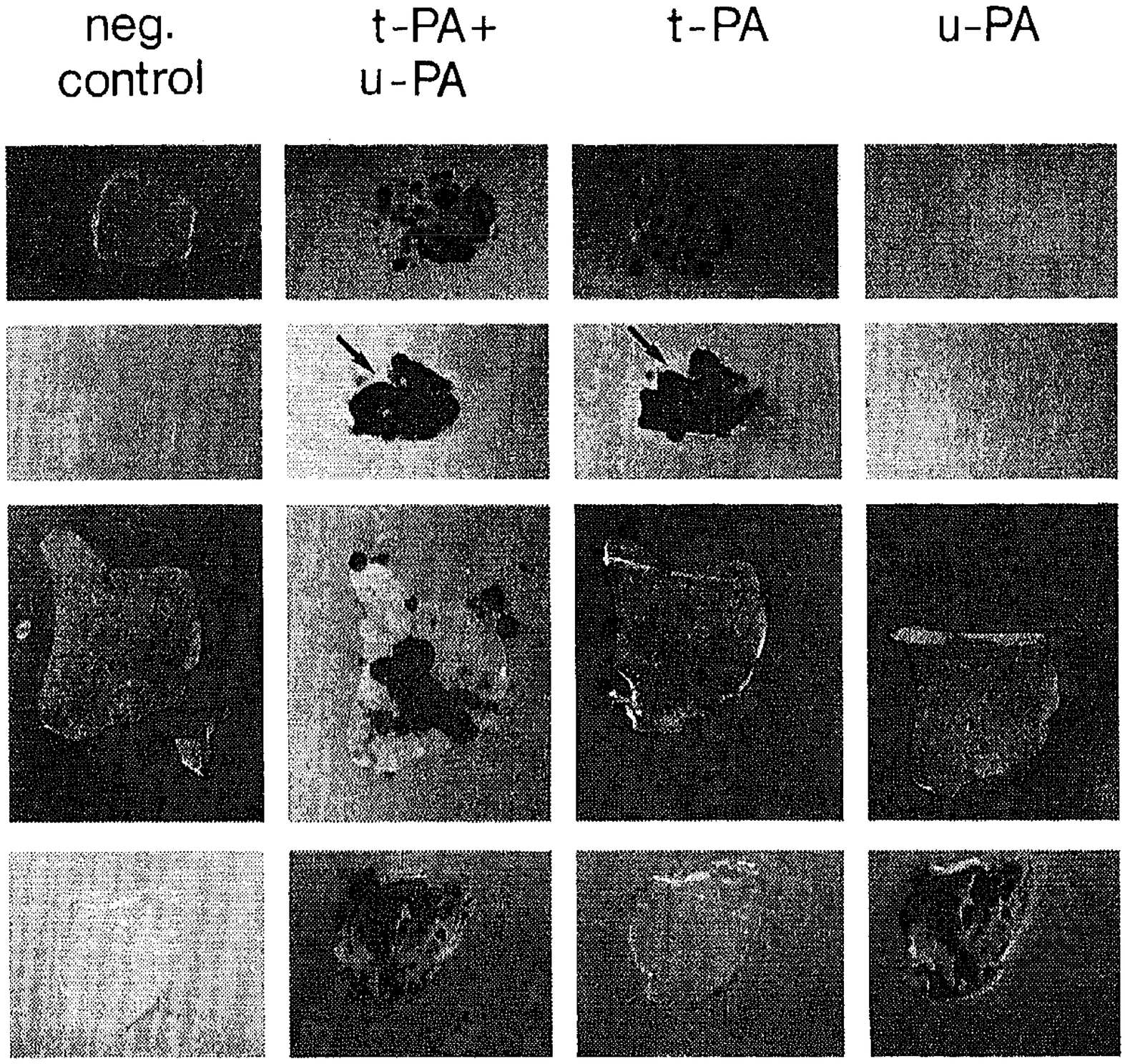

Fig. 3—*In situ* zymography on fresh uveal melanocytic lesions. Plasminogen activation was t-PA mediated in all cases studied and located in blood vessels and in tumour cells. Examples are shown of three cases. One non-spindle uveal melanoma had t-PA immunoreactivity in blood vessels but not in tumour cells (first row). One non-spindle uveal melanoma where 5–25 per cent of the tumour cells stained for t-PA in immunohistochemistry is shown (second row); note that lysis is more abundant at the sclera site (arrow). Also, a metastasis of uveal melanoma (third row) showed more generalized t-PA mediated lysis. A u-PA positive MV3 xenograft, where t-PA mediated lysis had not yet occurred, is included (fourth row). Columns: neg. control: casein layer without plasminogen; u-PA+t-PA: casein layer with addition of plasminogen; t-PA: casein layer+plasminogen+polyclonal antibody against u-PA; u-PA: casein layer+plasminogen+polyclonal antibody against t-PA. Incubation times at 37°C: 5 h in all uveal melanoma cases (top three rows) and 1 h (fourth row).

u-PA expression was found focally in only five primary uveal melanomas, all of which were of the non-spindle type. In two cases, u-PA expression correlated with TRD; one patient was known to have metastases; and for two patients, follow-up was relatively short. All metastases contained u-PA-positive cells. Therefore, a clear association with progression of disease exists, as could be found for some other types of tumours,[17,19–21] including cutaneous melanoma.[22] u-PA positivity might be an additional prognostic marker for uveal melanoma. Nevertheless, six cases with TRD did not display u-PA positivity. Therefore, although a relation between u-PA positivity and progression of disease could be demonstrated in this study, a more extensive study has to be performed to determine whether u-PA is a valuable prognostic marker for uveal melanoma.

With *in situ* zymography, we were able to detect only t-PA (both blood vessel and tumour cell associated) and no u-PA activity, which is in agreement with findings in primary cultures of uveal melanoma.[36] Apparently, the u-PA detected by immunohistochemistry is either of the inactive proform or inactivated by PAI. This view is supported by the fact that the staining was only cytoplasmic, where u-PA is normally in the inactive form. Also, the *in situ* zymography with regional sensitivity rather than cellular sensitivity might be too

insensitive to detect less than the 5 per cent scattered u-PA positive cells in the tumour.

The expression of u-PAR in many more tumours than u-PA is remarkable, though it is in agreement with our earlier findings in human melanoma cell lines,[16] where all cell lines, including the non-metastatic ones, expressed u-PAR. u-PAR was found in the cytoplasm, indicating that at least a portion of u-PAR was not available for binding to pro-u-PA.[10] Receptor bound pro-u-PA is more efficiently converted to active u-PA.[6–12]

For t-PA, u-PA, u-PAR and PAI-2, a higher expression (higher percentage of staining as well as a higher number of lesions involved) was found in the non-spindle cell uveal melanomas (Fig. 2) as compared with the spindle cell melanomas which have a better prognosis. These observations and the fact that these components were also frequently detected in metastases implicate the plasminogen activation system in the progression of disease. Surprisingly, although four out of five metastases were positive, in primary tumours this tendency could not be established for PAI-1, which is a prognostic marker for mammary carcinoma.[18]

By comparing the involvement of the PA system in uveal and cutaneous melanoma,[22] the following differences are noteworthy: (1) Using the same polyclonal antibody, t-PA was found in almost all primary and metastatic lesions of uveal melanoma, whereas in cutaneous melanoma, only a few metastases contained t-PA-positive tumour cells. (2) In cutaneous melanoma, lesions expressing u-PA also expressed u-PA's regulators u-PAR and PAI. For uveal melanoma, this co-expression was not as profound (Fig. 2). (3) Uveal melanomas expressed u-PA, PAI-1 and PAI-2 in approximately 10 per cent of the lesions, whereas in cutaneous melanoma approximately 60 per cent of the thicker primary lesions were positive. In addition, fewer tumour cells stained in uveal immunoreactive lesions (all u-PA-, PAI-1- and PAI-2 positive lesions were scored in the 1–5 per cent category). As in cutaneous melanoma, u-PA is associated with progression of disease in uveal melanoma, but in uveal melanoma, other proteolytic systems could be of more importance in metastatic spread, by comparison with cutaneous melanoma.

From our study, we conclude that in uveal melanoma, u-PA expression may be associated with metastatic disease and accordingly with a poor prognosis. In our opinion, it would be worthwhile to extend research on the presence of u-PA in uveal melanoma to a large group of tumours with accurate follow-up. In this way, it could be established whether u-PA positivity is of additional prognostic value in uveal melanoma.

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