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Plasminogen Activators, Their Inhibitors, and Urokinase Receptor Emerge in Late Stages of Melanocytic Tumor Progression

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Degradation of the extracellular matrix and other tissue barriers by proteases like plasminogen activators (PAs) is a prerequisite for neoplastic growth and metastasis. Recently, we reported that highly metastatic behavior of human melanoma cells in nude mice correlates with urokinase-type PA (u-PA) expression and activity and with PA inhibitor type 1 and 2 (PAI-1, PAI-2) expression. Here we report on the occurrence of components of the PA system in the various stages of human melanoma tumor progression* in situ. *We studied the protein distribution on freshly frozen lesions of common nevocellular nevi (n = 25), dysplastic (= atypical) nevi (n = 16), early primary melanomas (n = 8), advanced primary melanomas (n = 11), and melanoma metastases (n = 17). Tissue-type PA was present in endothelial cells in all lesions, whereas in metastases it could be detected in tumor cells in a minority of the lesions. u-PA, its receptor, PAI-1, and PAI-2 could not be detected in benign and in early stages but appeared frequently in advanced primary melanoma and melanoma metastasis lesions. u-PA was detected in stromal cells and in tumor cells at the invasive front, the u-PA receptor and PAI-2 in tumor cells, and PAI-1 in the extracellular matrix surrounding tumor cells. Localization of the corresponding messenger RNAs and enzyme activities revealed a similar distribution. We conclude that plasminogen activation is a late event in melanoma tumor progression. (Am J Pathol 1994, 144:70–81)

Migration of tumor cells is an essential step in metastasis. Tumor cells detach from a primary tumor, migrate through the surrounding tissue toward the circulation, and spread through the body. Eventually, they will invade a target tissue to form a distant metastasis. During several steps of this process, proteolytic degradation of the extracellular matrix is required. Different proteolytic enzyme systems are involved,[1,2] including the matrix metalloprotease system[3] and the plasminogen activation system.[4,5]

At least two distinct plasminogen activators (PAs) are known, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The activity of u-PA and t-PA can be modulated by two specific PA inhibitors, PAI-1 and PAI-2.[6] u-PA activity can be focused on the cell surface by a specific receptor (u-PAR).[7,8] Moreover, the u-PA activity can also be modulated by the interaction of u-PA with its receptor u-PAR because recent experiments have shown that binding to the receptor accelerates the conversion of pro-u-PA to active u-PA on the cell surface,[9,10] possibly by close interaction with the receptor-bound plasminogen.[11,12] Furthermore, the receptor ensures the availability of active u-PA on the cell surface by rapid internalization of inactive u-PA: PAI complexes.[13,14]

Elevated levels of u-PA have been reported for a number of human malignancies, including colon,[15–17] breast,[18,19] urinary bladder,[20] and prostate cancer.[21] For the colon, we have reported before that the sequential steps in colon tumor progression, ie, adenomatous polyp, adenoma with atypia, and ad-

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enocarcinoma, is reflected by discrete stepwise increase of u-PA.[17] u-PA antigen levels as estimated in tissue sections and by enzyme-linked immunosorbent assay might be used as prognostic markers in colon and breast cancer.[17–19,22] The actual involvement of PAs in tumor cell migration or metastasis has been demonstrated in several model systems.[23–27] Localization studies have been performed in several malignancies using immunohistochemistry or RNA *in situ* hybridization. A rather heterogeneous pattern of distribution was found. Surprisingly, u-PA was not only found in tumor cells,[28–30] but also in stromal cells surrounding the tumor cells,[31,32] depending on the malignancy studied. u-PAR was found to be tumor cell-associated,[32] PAI-1 endothelial cell-[33] or tumor cell-associated.[29,34]

We recently demonstrated, using a nude mouse model system, that in human melanoma cell lines differing in metastatic potential, u-PA, PAI-1, and PAI-2 could only be found in those cell lines that were able to form lung metastases after subcutaneous inoculation in nude mice.[35] The nonmetastatic cell lines did not produce u-PA, PAI-1, or PAI-2 but produced only t-PA. However, little is known about the involvement of the plasminogen activation system in fresh human cutaneous melanocytic lesions.[36,37]

In the present study, different stages in melanoma tumor progression, common nevocellular nevi, dysplastic (= atypical) nevi, early and advanced primary melanoma, and melanoma metastases were analyzed for the occurrence of the different components of the plasminogen activation system. The presence and distribution of protein, messenger (m)RNA, and enzymatic activity were studied using immunohistochemistry, RNA *in situ* hybridization, and *in situ* zymography on tissue sections.

Materials and Methods

Tissue Specimens

Representative tissue samples were freshly received from cutaneous melanocytic lesions excised from patients at the University Hospital, Nijmegen, The Netherlands, snap-frozen in liquid nitrogen, and stored at −70 C until sectioning. Based on conventional histopathological examination on paraffin sections, lesions were divided into five classes: common nevocellular nevus (n = 25), dysplastic (atypical) nevus (n = 16), early primary melanoma (Breslow thickness <1.5 mm, n = 8), advanced primary melanoma (Breslow thickness ≥1.5 mm, n = 11), and distant melanoma metastasis (n = 17). Other frozen tissue specimens, used for the characterization of the antibodies, were from the archives of the Pathology Department.

Antibodies

Rabbit anti-human t-PA and u-PA polyclonal antibodies have been used in earlier work.[35,38] Monoclonal antibodies against human t-PA[39] were kindly provided by R. Bos (Gaubius Laboratory IVVO-TNO, Leiden, The Netherlands). Monoclonal antibodies against human u-PA (≠3689), human u-PAR (≠3936), and human PAI-1 (≠380)[40] were purchased from American Diagnostica Inc. (Greenwich, CT). The rabbit and goat polyclonal antibodies against human PAI-2 were a generous gift from E. Schüler (Behring Werke AG, Marburg, Germany). A polyclonal antibody against human heparan sulphate proteoglycan[41] was kindly provided by L. van den Heuvel (Department of Biochemistry, Nijmegen University, Nijmegen, The Netherlands).

Characterization of Anti-t-PA and Anti-PAI-2 Antibodies for Use in Immunohistochemistry

Monoclonal antibody 5.1 against human t-PA was incubated with Western blot strips containing purified human t-PA and a cell lysate of a human melanoma cell line 1F6.[35] The antibody recognized t-PA and did not cross-react with proteins of the lysate. We characterized further the antibody for its use in immunohistochemistry on fresh tissue sections of 20 different human organs. Blood vessel endothelium stained intensely in all tissues studied, and the staining pattern compared well with the staining pattern obtained with the polyclonal antibody. Two polyclonal antibodies against human PAI-2 were tested on Western blot strips containing recombinant PAI-2 and 1F6 cell lysate. Both polyclonal antibodies recognized PAI-2, and neither of the two cross-reacted with proteins of the cell lysate. In immunohistochemistry, the antibodies stained syncytiotrophoblasts in sections of fresh postnatal placenta lesions.

Immunohistochemistry

For immunohistochemistry, 4-μ cryostat sections were air-dried overnight at room temperature and stored at −80 C until use. Before incubation with the primary antibody, sections were fixed for 10 minutes in acetone at −20 C. For monoclonal antibodies, a three-step avidin biotin peroxidase complex method was applied (Vectastain Elitekit, Vector Laboratories, Burlingame CA). Dilutions of antibodies were in phosphate-buffered saline (PBS), pH 7.4, with 1%

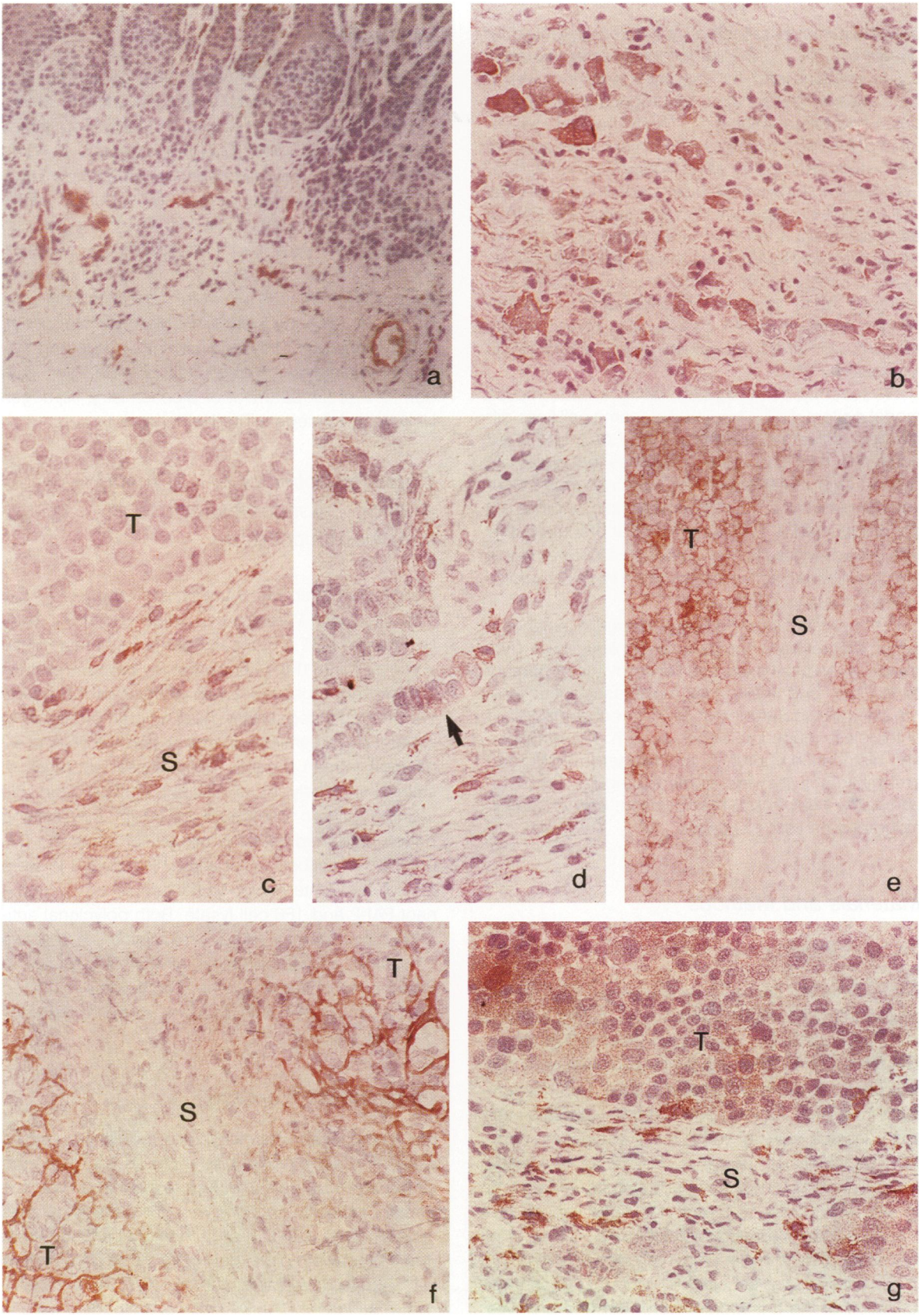
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Table 1. *Immunohistochemical Staining of the Components of the Plasminogen Activation system in Advanced Primary Malignant Melanomas and Metastases of Melanoma*

	t-PA	u-PA	u-PAR	PAI-1	PAI-2
Lesion					
Advanced primary melanoma, Breslow ≥ 1.5 mm					
Case 1	–	–	–	–	–
Case 2	–	–	–	–	–
Case 3	–	–	–	t	–
Case 4	–	–	–	–	–
Case 5	–	t, s	t, s	t	t
Case 6	–	–	–	–	–
Case 7	e	t, s	t,	e	t
Case 8	–	t	t	e	t
Case 9	–	t, s	t, s	e	t
Case 10	–	t, s	t	e	t
Case 11	e	t, s	t	e	t, s
Melanoma metastasis					
Case 12	t	t, s	–	–	–
Case 13	–	t, s	–	e	–
Case 14	–	–	–	–	–
Case 15	–	t, s	t	e	t
Case 16	t	t, s	t, s	t, s	t
Case 17	t	t, s	s	e	t
Case 18	t	s	t	–	t, s
Case 19	–	t, s	t, s	t, s	s
Case 20	t	t, s	t	e	–
Case 21	–	–	t	–	–
Case 22	t	t, s	t	–	t, s
Case 23	–	–	–	–	–
Case 24	–	t, s	t	s	–
Case 25	–	t, s	–	s	t, s
Case 26	–	–	–	–	–
Case 27	–	s	s	s	s
Case 28	–	–	t	–	–

t = staining of tumor cells; e = extracellular matrix stained in these lesions; s = stromal cells stained in these lesions; – = no staining observed.

bovine serum albumin and all incubations took place in a humidified chamber at room temperature. Monoclonal antibodies were applied in a concentration of 10 μg/ml, except for monoclonal antibody 5.1 (50 μg/ml). After incubation with the monoclonal antibody for 60 minutes, sections were washed three times for 5 minutes in PBS, and biotinylated goat anti-mouse immunoglobulin was added for 30 minutes. Subsequently, the slides were washed again three times for 5 minutes in PBS, the avidin biotin peroxidase complex was added for 45 minutes, and sections were washed three times for 5 minutes in PBS.

Polyclonal antibodies were applied on the sections for 60 minutes, washed three times for 5 minutes in PBS, incubated with peroxidase-labeled goat anti-rabbit immunoglobulin for 30 minutes, and washed again three times for 5 minutes in PBS. Bound antibodies were visualized by using 3-amino-9-ethylcarbazole as a substrate for peroxidase. After counterstaining with Meyer's hematoxylin, sections were mounted with Kaisers glycerin (Merck, Darmstadt, Germany). An incubation where the first antibody was omitted served as a negative control. For each section, the percentage of positive melanocytic cells was estimated. Each section was assigned to one of the following categories: 0%, 1 to 5%, 5 to 50% and 50 to 100% positivity. Notes were taken of other staining components (eg, fibroblastlike cells, extracellular matrix) among the melanocytic areas.

Probes and Labeling

For the RNA *in situ* hybridization experiments, DNA probes were used applying the following complementary (c)DNA fragments:[42] a 2.2-kb *Kpn*I-*Hind*III fragment of human t-PA, a 1.0-kb *Eco*RI-*Hind*III fragment of human u-PA, a 1.3-kb *Bam*HI fragment

Figure 1. *Immunohistochemistry of components of the plasminogen activation system in fresh human melanocytic lesions, typical examples are shown.* **a:** *t-PA protein in blood vessels of a nevocellular nevus.* **b:** *t-PA present in tumor cells of melanoma metastasis lesion.* **c** *and* **d:** *u-PA is present in fibroblastlike cells in the stroma (S), not in the tumor (T), and occasionally in invading tumor cells (arrow) in this primary melanoma lesion.* **e:** *u-PAR is located in the membrane of tumor cells in this primary melanoma.* **f:** *Networklike PAI-1 distribution in the extracellular matrix surrounding tumor cells in a melanoma metastasis lesion.* **g:** *PAI-2 was found in tumor cells and in fibroblastlike cells of the stroma in this primary melanoma. Magnifications:* **a, e:** *600×*, **b, c, d, f, g:** *750×*.

of human u-PAR, a 2.5-kb *Eco*RI fragment of human PAI-1, and a 0.9-kb *Eco*RI-*Hind*III of human PAI-2. cDNA coding for ribosomal RNA and phage λ *Hind*III fragments were used as positive and negative controls. The cDNAs were labeled with [^{35}S]dCTP (600 Ci/mmol, Amersham International, Amersham, UK) by random priming (random primed DNA labeling kit, Boehringer, Mannheim, Germany). Finally, the probes were purified by gel filtration on Sephadex G-50 and phenol extraction. The probes were stored at −20 C until use.

RNA in Situ *Hybridization*

For *in situ* hybridization, 8-μ frozen sections were mounted on organo-silane-coated slides and fixed for 5 minutes in 4% PBS-buffered paraformaldehyde. The sections were washed in PBS and stored in 70% ethanol at 4 C until use. The hybridization procedure was adapted from published procedures.[43,44] Briefly, sections were rinsed three times in 2× sodium dodecyl sulfate (SSC). Acetylation was performed for 10 minutes in 0.1 mol/L triethanolamine, pH 8.0, containing 0.25% acetic acid anhydride. The slides were rinsed briefly in 2× SSC and in PBS and incubated for 30 minutes in 0.1 mol/L TrisHCl, pH 7.0, supplied with 0.1 mol/L glycine. Finally, the sections were rinsed in 2× SSC for 10 minutes and dehydrated in ethanol. Heat-denatured [^{35}S]dCTP-labeled probe was added to the hybridization mixture (50% formamide, 2× SSC, 10% dextran sulphate, 0.1% bovine serum albumin, 0.1% polyvinylpirrolidone, 0.1% ficoll 400, 1 mg/ml yeast transfer RNA and 10 mmol/L dithiothreitol (DTT) at a concentration of 2 × 10^5 dpm per section. The mixture was applied on the sections and spread equally by a coverslip. Before hybridization, sections were put in a humidified box at 65 C for 10 minutes. Hybridization was performed overnight in a humidified box at 42 C. After hybridization, the coverslips were removed by submerging in a solution of 2× SSC, 50% formamide with 10 mmol/L DTT. The slides were then washed for 45 minutes in 2× SSC in 50% formamide supplied with 10 mmol/L DTT at 42 C. Two washes of 45 minutes in 1× SSC, 50% formamide with 10 mmol/L DTT at 42 C and one wash of 45 minutes in 0.5× SSC, 50% formamide with 10 mmol/L DTT at 42 C followed. The sections were quickly rinsed in 1× SSC, dehydrated in alcohol, and air-dried. Subsequently, the sections were dipped in Kodak NTB2 film emulsion and autoradiographed for 7 or 14 days at 4 C. The emulsion was developed in Kodak D19 developer and fixed in 24% sodium thiosulphate. Finally, the sections were counterstained by hematoxylin or by hematoxylin and eosin and were mounted with synthetic resin.

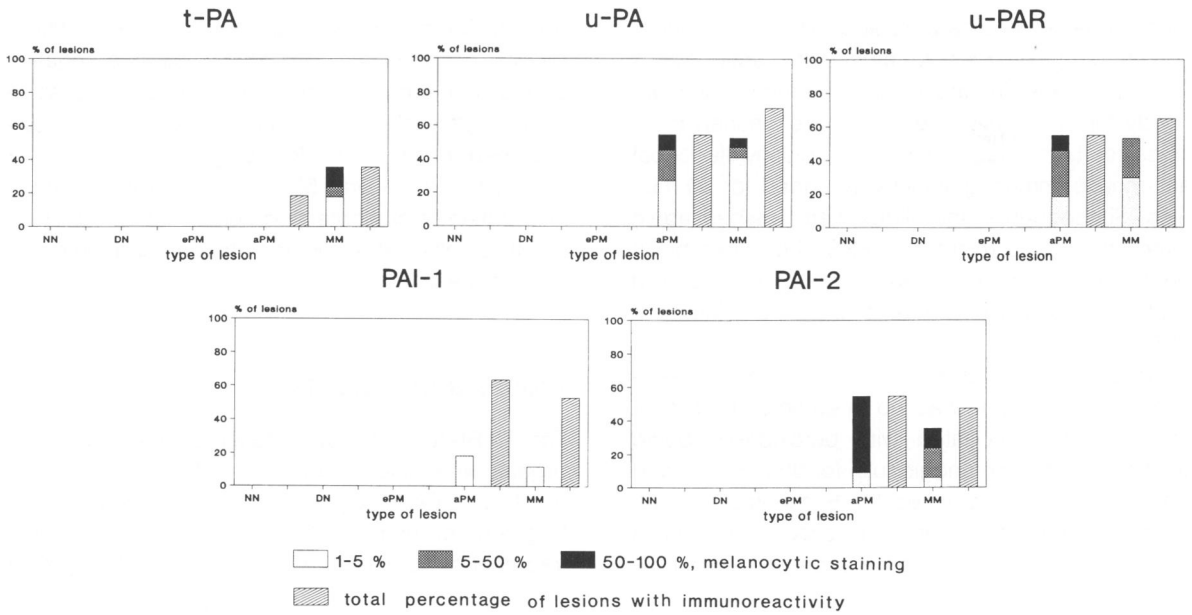


Figure 2. *Percentage of immunobistochemically stained melanocytic cells and percentage immunoreactive lesions including staining of extracellular matrix and stroma cells (e and s in Table 1), expressed as percentage of the total number of lesions. NN = nevocellular nevus (n = 25), DN = dysplastic nevus (n = 16), ePM = early primary malignant melanoma with Breslow thickness <1.5 (n = 8), aPM = advanced primary malignant melanoma with Breslow thickness ≤1.5 (n = 11), MM = melanoma metastasis (n = 17). For t-PA, the results with the polyclonal antibody are shown, for u-PA, the results with the monoclonal antibody, and for PAI-2, the results obtained with the goat polyclonal antibody are shown. Results with the other t-PA, u-PA, and PAI-2 antibodies were comparable. See also Table 1.*

In Situ *Zymography*

Cryostat sections were covered with an overlay mixture containing 2% (w/v) commercial instant nonfat dry milk solution (an 8% [w/v] stock solution in PBS was heated at 95 C for 30 minutes, briefly centrifuged at $3 \times 10^3 g$, the supernatant was used), 0.9% agar (w/v), and 30 μg/ml plasminogen as described by Sappino et al.[43] The overlay solution was prepared at 50 C, and 130 μl was applied on freshly defrosted 8-μ sections, spread evenly under 24 × 32-mm glass coverslips. Slides were incubated at 37 C in a humidified air box. Development of lysis was allowed for up to 20 hours, with photography at intervals. Experiments with addition to the overlay mixture of 2 mmol/L amiloride or polyclonal antibody against u-PA to inhibit u-PA activity or with addition of polyclonal antibody against t-PA to specifically inhibit t-PA activity were performed to discriminate between the two types of plasminogen activation activities.

Results

Protein Localization

Tissue sections of nevocellular nevi, dysplastic nevi, primary melanoma, and melanoma metastases were stained for t-PA, u-PA, u-PAR, PAI-1, and PAI-2. Typical examples of immunohistochemical staining of all PA components are shown in Figure 1. No immunohistochemical staining of melanocytic cells for any of the five PA components was observed in any benign or premalignant lesions studied (common nevocellular nevi and dysplastic nevi) nor in the early primary melanoma lesions. Staining of tumor cells for u-PA, u-PAR, PAI-1, and PAI-2 was observed in a number of advanced primary melanoma and melanoma metastasis lesions. Tumor cells expressing t-PA were observed in a few melanoma metastases only (Table 1). Although u-PA, u-PAR, PAI-1, and PAI-2 were frequently detected, the percentage of immunoreactive cells varied in the different lesions (Figure 2). Immunoreactivity for u-PA, u-PAR, and PAI was mostly encountered in the same lesion (Table 1).

In all stages of melanoma tumor progression, t-PA immunoreactivity was found in endothelial cells of blood vessels (Figure 1a), but immunoreactivity of tumor cells was restricted to melanoma metastases only (Figures 1b and 2). The results obtained with both polyclonal and monoclonal antibodies corresponded.

In general, u-PA immunoreactivity was confined to stromal, fibroblastlike cells (Figure 1, c and d) and a minority of tumor cells (1 to 5% category) in primary melanoma and melanoma metastasis lesions (Figure 2, Table 1). Stained tumor cells were often localized at the tumor stroma interface at the invasive front (Figure 1d). Results with monoclonal antibody #3689 were identical to the results obtained with the polyclonal antibody.

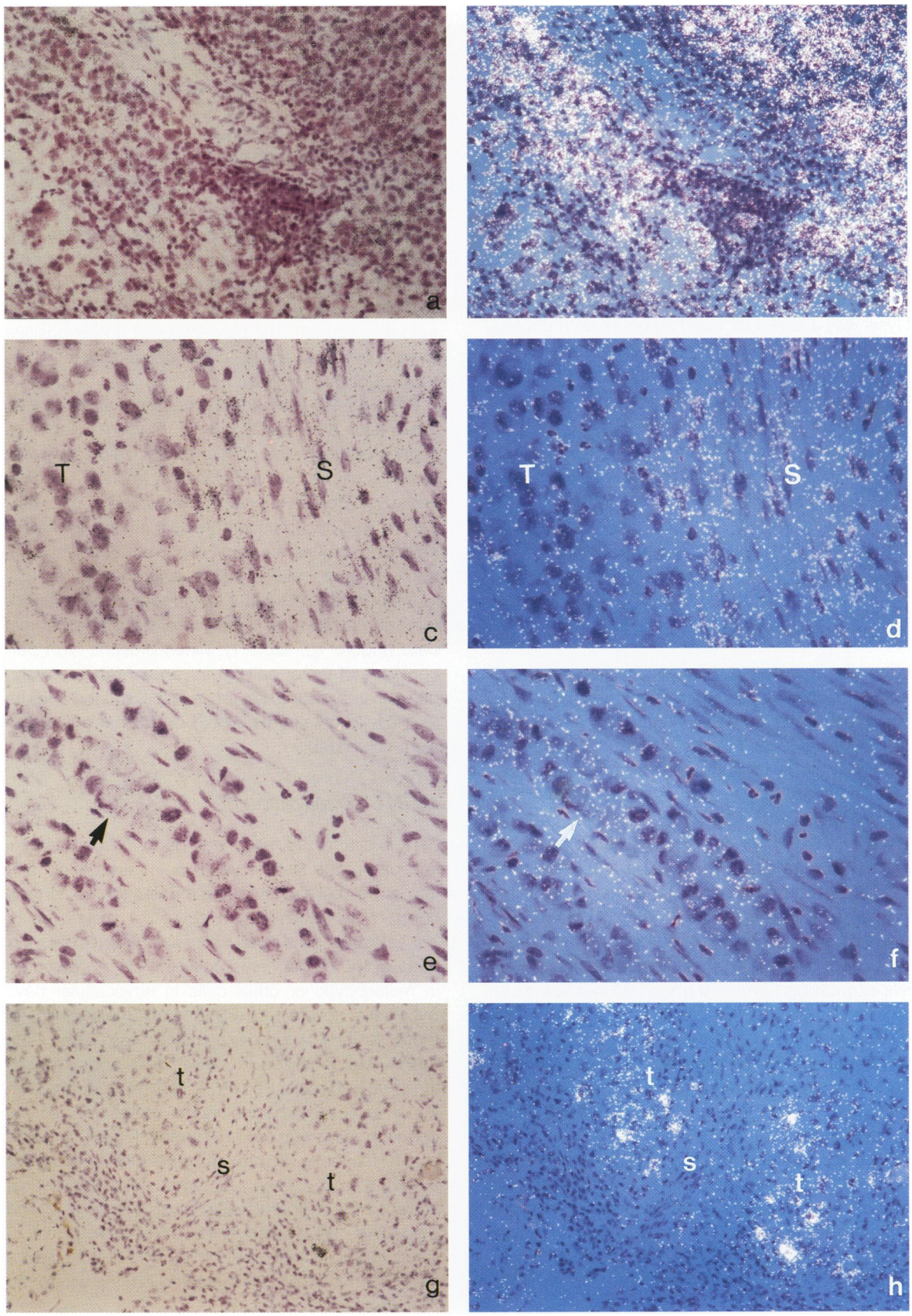
u-PAR protein was found in the same lesions as u-PA protein. u-PAR immunoreactivity was mainly membrane associated and was observed primarily in tumor cells (Figure 1e). Occasionally, stromal cells also stained. The percentage of u-PAR-positive tumor cells was similar to the percentage of u-PA-positive cells (Figure 2).

PAI-1 protein was mainly localized in the extracellular matrix around tumor cells (Figure 1f, Table 1). This localization was confirmed by staining parallel tissue sections with a polyclonal antibody against heparan sulphate proteoglycan, which is an extracellular matrix component (results not shown). More scarcely, immunoreactive PAI-1 was found in a minority of tumor cells (1 to 5% category, Figure 2).

In contrast to the PAI-1 localization, the PAI-2 protein was localized in tumor cells and some stromal cells (Figure 1g, Table 1). When present, high percentages of tumor cells in advanced primary melanomas and melanoma metastases stained (Figure 2). Results obtained by both polyclonal antibodies were comparable.

mRNA Localization

RNA *in situ* hybridization was performed on parallel sections of the lesions used for immunohistochemistry to establish whether the mRNAs for the components of the plasminogen activation system could be assigned to similar cells as the proteins. For none of the components could mRNA be detected in melanocytic or other cells in nevocellular nevi, dysplastic nevi, and early primary melanomas. In those lesions where expression was found, the mRNA was localized in similar cells and areas as the protein (Figure 3). t-PA mRNA was localized in tumor cells of some melanoma metastasis lesions corresponding with the t-PA protein (Figure 1, a and b). As for the u-PA protein, u-PA mRNA could be detected in a minority of tumor cells (results not shown) and in fibroblastlike cells (Figure 3, c and d). u-PAR, PAI-1, and PAI-2 mRNAs were exclusively found in tumor cells (Figure 3, e to h, results for PAI-2 not shown) in the same lesions where the protein could be detected.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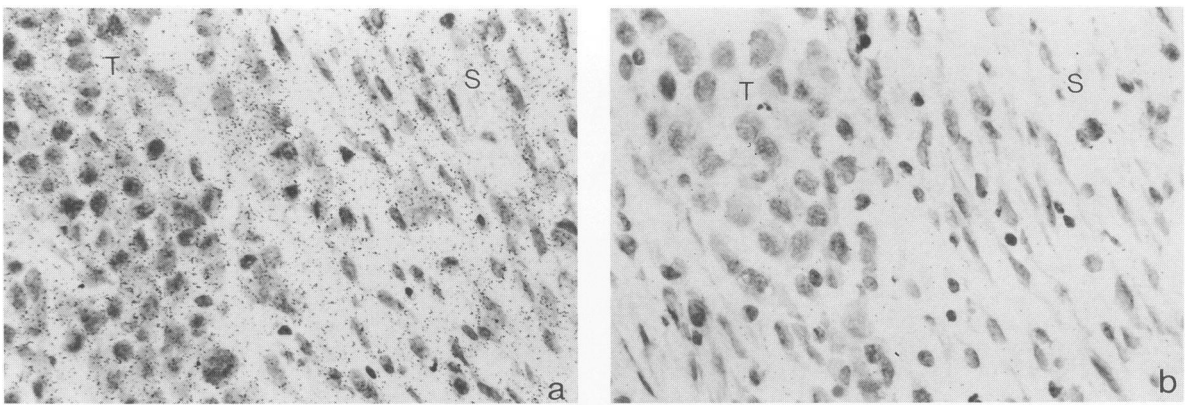

Figure 4. *RNA* in situ *hybridization: positive and negative controls. Sections of the same malignant melanoma specimen as shown in Figure 3* c *to* f *were hybridized with cDNA probe for ribosomal RNA* (a) *or with labeled phage* λ *DNA fragments* (b). *Abbreviations: S = stroma, T = tumor. Magnification: 1200×; exposure time: 1 week.*

Using a ribosomal RNA probe as a positive control for the hybridization, we found the signal localized in tumor cells as well as in fibroblastlike cells (Figure 4a). Labeled phage λ fragments were used as a negative control for the hybridization and did not hybridize to any cell type (Figure 4b).

Localization of Plasminogen Activation

In situ zymography was performed on sections of four nevocelluar nevi, six dysplastic nevi, six advanced primary melanomas, and seven melanoma metastases. In all lesions studied, the plasminogen activation pattern corresponded with the immunohistochemical staining. Plasminogen activation was t-PA-mediated in nevocellular and dysplastic nevi and could be assigned, by comparison with the hematoxylin and eosin section, to areas with blood vessels. In the advanced primary melanomas, plasminogen activation was u-PA-mediated and could be assigned to groups of tumor cells or stromal areas. When no immunohistochemical staining of u-PA or t-PA was observed, the same lesion did not display plasminogen activation. In one advanced primary melanoma, areas with both t-PA- and u-PA-mediated plasminogen activation were observed (Figure 5). In tumor cells of melanoma metastases, both t-PA- and u-PA-mediated plasminogen activation was detected (Figure 5). Apart from t-PA-mediated plasminogen activation in blood vessels, plasminogen activation in malignant lesions was always in areas with tumor cells.

Discussion

In a recent study, we investigated the involvement of the plasminogen activation system in six human melanoma cell lines. u-PA, PAI-1, and PAI-2 mRNA and protein levels were elevated in cell lines that highly metastasized in nude mice. Furthermore, matrix degradation by these cell lines was u-PA-mediated.[35] In the current study, we investigated the presence of components of the plasminogen activation system in different stages of cutaneous melanoma tumor progression.

We found t-PA protein and activity present in blood vessels of benign, premalignant, and malignant lesions. All other components, however, were expressed only in the most malignant lesions: u-PA protein, mRNA, and activity were observed only in advanced primary melanomas and in melanoma metastases; u-PAR, PAI-1 and PAI-2 proteins, and mRNA were also restricted to advanced primary melanomas and melanoma metastases. Mostly, u-PA, u-PAR, and PAI proteins were co-expressed in the same lesion. t-PA protein, mRNA, and activity was observed in tumor cells of some of the metastasis lesions only.

Although in an earlier study, t-PA was detected immunohistochemically in all five primary melano-

Figure 3. *RNA* in situ *hybridization of components of the plasminogen activation system in fresh human melanocytic lesions. Examples are shown obtained from sections from the same specimen as the immunohistochemistry examples shown in Figure 1.* a, b: *t-PA mRNA present in tumor cells of this melanoma metastasis lesion.* c, d: *u-PA mRNA present in fibroblastlike cells in the stroma of this primary melanoma lesion.* e, f: *u-PAR mRNA is present in a ridge (arrow) of tumor cells in this primary melanoma lesion.* g, h: *PAI-1 mRNA is present in two groups of tumor cells in a melanoma metastasis. No signal is present in the separating stroma nor in the lymphocytes present in this lesion.* b, d, f *and* h *are darkfield images of* a, c, e *and* g *respectively. Abbreviations: S = stroma, T = tumor; magnifications:* a, b, g, h: *600×,* c *to* f: *1200× exposure time: 1 week* (c *to* h) *or 2 weeks* (a, b).

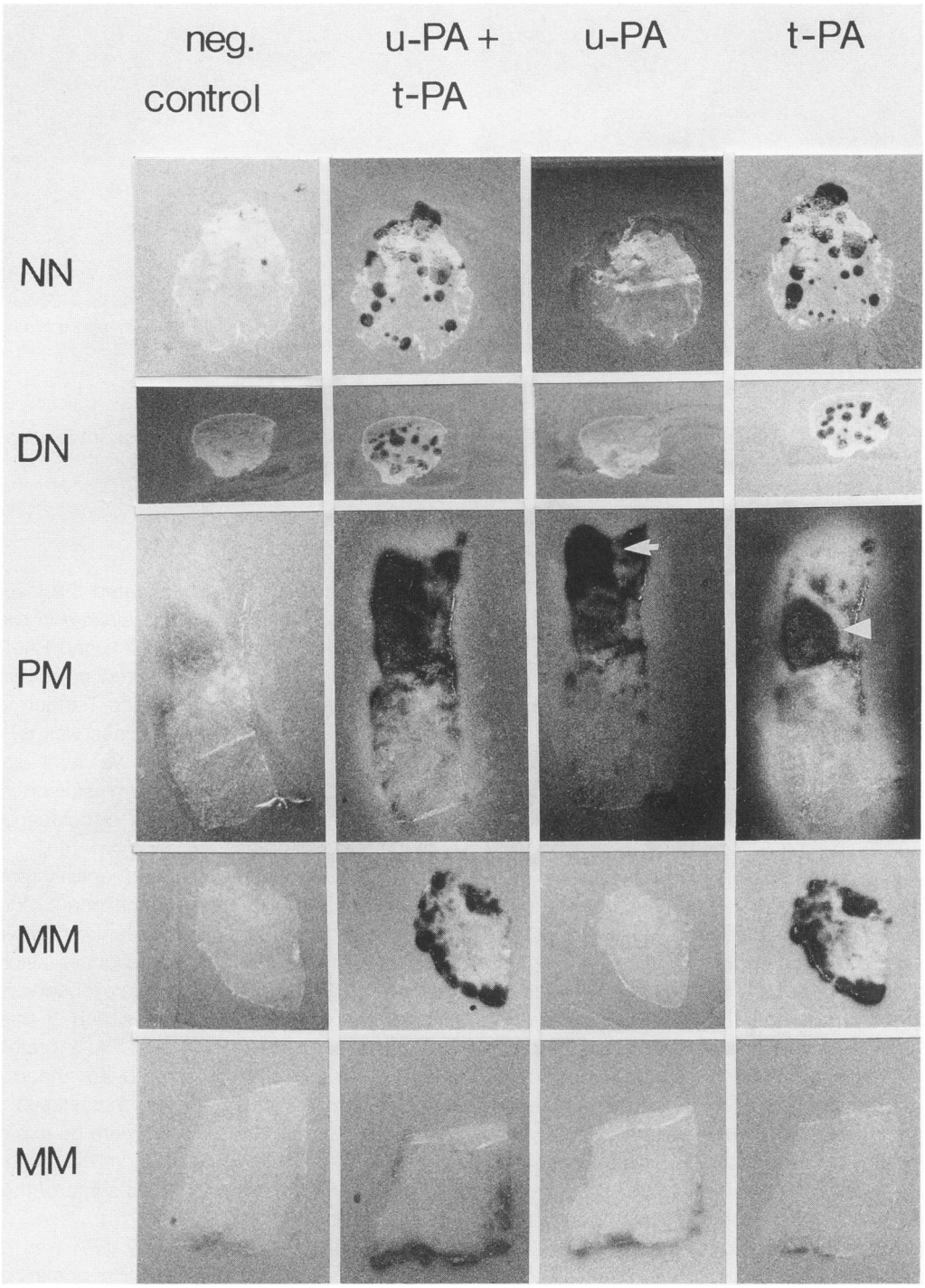

Figure 5. In situ *zymography on fresh melanocytic lesions. t-PA-mediated plasminogen activation in blood vessels in nevocellular nevus (NN) and dysplastic nevus (DN). Tumor cell-associated plasminogen activation was encountered in one advanced primary melanoma with areas of t-PA- (arrowhead) and u-PA- (arrow) mediated plasminogen activation and in two metastases of melanoma (MM) with t-PA-mediated plasminogen activation (fourth row) and u-PA-mediated plasminogen activation (fifth row). Columns: neg. control: casein layer without plasminogen, u-PA + t-PA: casein layer with addition of plasminogen; u-PA: casein + plasminogen + polyclonal antibody against t-PA; t-PA: casein + plasminogen + polyclonal antibody against u-PA, which shows similar results as casein + plasminogen + amiloride (not shown). Magnification: 4×. Incubation times at 37 C: 60 minutes (MM case 5), 90 minutes (MM case 13), 150 minutes (NN and DN), and 18 hours (PM).*

mas, all five metastases, and all four cell lines and derived xenografts analyzed,[37] we now demonstrate that t-PA can only be detected in metastases. We used two different antibodies against t-PA on 77 melanocytic lesions, and the staining pattern of the two antibodies corresponded in all lesions. Moreover, the use of two complementary techniques (mRNA *in situ* hybridization and *in situ* zymography) confirmed our histochemical findings. The differences found could be due to differences in sensitivity of the antibodies used.

From recent literature, the view emerges that in various malignancies, neoplastic[12,15,16,28–30] as well as bordering stromal (fibroblastlike) cells[31,32] are producers of u-PA. Markus et al[36] previously visualized u-PA protein in a few primary melanoma sections. They found strong staining of u-PA at the cell membranes of tumor cells and weak staining of stromal cells. We located u-PA mainly to stromal cells, with a minority of u-PA-positive tumor cells at the invasive front.

With the novel antibody #3936 against u-PAR in our study, we mainly stained tumor cells and occasionally a few stromal cells in all lesions where u-PA protein was detected. At the cellular level, differences exist in distribution of u-PA and u-PAR. u-PA is mainly located in fibroblastlike cells and in a minority of tumor cells, u-PAR is almost exclusively present in tumor cells and almost absent in stromal cells. This could suggest that either fibroblastlike cells secrete pro-u-PA that is bound to the invading tumor cells or u-PA is provided by tumor cells in an autocrine fashion.

PAI-1 protein was localized in extracellular matrix surrounding nests of tumor cells that contained the mRNA. This is different from the situation in colon,[33] where mRNA is produced by endothelial cells in the vicinity of the invasive front, but in agreement with squamous cell carcinoma[29] and breast cancer.[34] Recent *in vitro* studies confirmed the attachment of free PAI-1 to the extracellular matrix.[45] Particularly, the extracellular matrix component vitronectin was found to be involved in the regulation of plasminogen activation *in vitro* as it might down-regulate u-PA[46] and binds plasminogen and PAI-1.[47]

A high percentage of tumor cells was found positive for PAI-2 protein. This could indicate a higher total expression level of PAI-2 relative to PAI-1 in these lesions, which was also found by enzyme-linked immunosorbent assay experiments for colorectal carcinoma[22] and gastric carcinoma.[48] However, it is probable that the importance of PAI-2 relative to PAI-1 in PA inhibition is less, as it is hardly secreted by cells.[49] We also localized PAI-2 intracellularly, whereas the localization of PAI-1 was extracellular.

We previously reported a strong correlation between metastasis of human melanoma cells in the nude mouse model and expression of u-PA and PAI.[35] In the present study using fresh human cutaneous melanocytic lesions, it is shown that u-PA, u-PAR, PAI-1, and PAI-2 appear in late stages of melanocytic tumor progression. Also, in this case, it is striking that u-PA expression coincides with the expression of their regulators, u-PAR and PAI-1 and PAI-2.[22,34] Heterogeneity of expression of PA components among advanced primary melanomas (in four out of 11 thick melanomas, no staining of components of the PA system) could be of great interest. Analogous to breast cancer,[18,19] it could well be that PA status could become an additional prognostic factor for progression of melanoma next to established histological criteria such as Breslow thickness[50] or Clark's level of invasion.[51] Therefore, we feel that a study on PA components in a large series of melanoma lesions is warranted.

Acknowledgments

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