Metastatic Behavior of Human Melanoma Cell Lines in Nude Mice Correlates with Urokinase-Type Plasminogen Activator, its Type-1 Inhibitor, and Urokinase-mediated Matrix Degradation

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Abstract. Five out of six human melanoma cell lines tested were able to degrade in vitro a smooth muscle cell extracellular matrix in a plasmin-dependent way. In three of these five cell lines, this process was mediated by tissue-type plasminogen activator (t-PA) and in the other two cell lines by urokinase-type plasminogen activator (u-PA).

All melanoma cell lines produced t-PA mRNA and protein, whereas only the two cell lines showing u-PA-mediated matrix degradation produced u-PA mRNA and protein. These latter cell lines also produced plasminogen activator inhibitor type-1 (PAI-1) and type-2 (PAI-2) mRNA and protein. u-PA receptor (u-PA-R) mRNA and binding of radiolabeled u-PA was found in all melanoma cell lines. The metastatic capacity of these cell lines was studied in nude mice. All cell lines were able to develop primary tumors at the subcutaneous inoculation site. The production of plasminogen activators, their inhibitors and urokinase receptor by subcutaneous tumors corresponded with the production by the parental cell lines in vitro.

The two u-PA and PAI-1 producing cell lines showed the highest frequency to form spontaneous lung metastases after subcutaneous inoculation, whereas five of the six cell lines formed lung colonies after intravenous inoculation.

In conclusion, u-PA mediated matrix degradation in vitro and production of u-PA and PAI-1 by human melanoma cell lines correlated with their ability to form spontaneous lung metastasis in nude mice. No correlation was found with the ability to form lung colonies after intravenous injection. These findings suggest a role for u-PA and PAI-1 in a relatively early stage of melanoma metastasis.

During metastasis, tumor cells must penetrate basement membranes and interstitial tissues, when they detach from the primary tumor and intravasate into the circulation, and later when they extravasate at the site formation of the secondary tumor. This means that these tumor cells should express the right panel and adequate levels of proteolytic enzymes to degrade the extracellular matrix (15, 16, 24, 34, 49, 57). In addition, these enzymes may have a function in the process of angiogenesis when endothelial cells grow invasively into the newly formed tumor and form new blood vessels (25, 38). The serine protease plasmin is one of the major enzymes believed to be involved in such proteolytic processes (15, 16, 24, 42, 43). Plasmin has a broad substrate specificity and can digest most of the components of the extracellular matrix including the basement membrane, either directly or by activation of proenzymes of metalloproteinases, like type IV collagenase or interstitial collagenase (23, 39, 57). Plasmin is formed by a conversion of the zymogen plasminogen, which is regulated by plasminogen activators. Two distinct plasminogen activators are known, the tissue-type (t-PA), and the urokinase-type (u-PA). The activity of the activators can be regulated by interactions with specific inhibitors, of which two have been described, type 1 (PAI-1) and type 2 (PAI-2) (33, 53). In addition, u-PA and its proenzyme, pro-u-PA, can be localized at cell surfaces by binding through their growth factor domain to a specific receptor (u-PA-R) (2, 8, 12, 41, 51, 55, 56, 62).

To study the role of plasminogen activation system in metastasis of malignant melanomas, we have investigated the occurrence of its various components and their mRNA in a set of six human melanoma cell lines with different metastatic behavior in the nude mice. To study the different steps in the metastatic process (primary tumor growth, local invasion and detachment of tumor cells from the primary tumor, lodgement and invasion in distant tissues) the cells were inoculated either subcutaneously or intravenously.

1. Abbreviations used in this paper: PAI, plasminogen activator inhibitor; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; u-PA-R, urokinase plasminogen activator receptor.
Furthermore, the ability of tumor cells to degrade extracellular matrix produced by smooth muscle cells was used as an in vitro model in which the effects of anticalcitropic antibodies against t-PA and u-PA were tested.

**Materials and Methods**

**Cell Lines**

All cell lines were derived from human melanoma metastases. The IF6 (59) and MV3 (60) cell lines were developed from lymph node metastases of two different male patients. The BLM cell line is a subline of BRO (35) and was isolated from lung metastases after subcutaneous inoculation of nude mice with BRO cells. The M14 (31) cell line was obtained from Dr. A. Cochran (John Wayne Clinic, Johnsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA). The Mel57 (9) cell line was kindly provided by Dr. J. de Vries (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The 530 cell line (64) was established and kindly provided by Dr. P. I. Schrier (University Hospital, Leiden, The Netherlands).

**Cell Culture**

All cell lines were grown as monolayers in DME medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To determine the protein and mRNA levels of plasminogen activators and their inhibitors fresh serum containing medium was added to the cell cultures shortly before they reached confluency, 24 h later when the cells had reached confluency, the conditioned medium was collected and cell extracts were prepared for antigen determination and RNA isolation.

**Assay for PA and PAI Antigen**

Antigen levels of t-PA, u-PA, PAI-1, and PAI-2 were determined in both conditioned media and cell extracts (extracted with 0.5% Triton X-100 and scraped with a rubber policeman) of cultured human melanoma cell lines. The antigen levels in tissue extracts of subcutaneous tumors derived from these cell lines after inoculation in nude mice were also determined. Extracts of subcutaneous tumors in nude mice were prepared using 0.1 M Tris- HCl (pH 7.5)/0.1% Tween 80 as a homogenization buffer. The final extracts contained 50 mg tumor tissue/μl. The antigen levels in tissue extracts of subcutaneous tumors derived from these cell lines after inoculation in nude mice were also determined. Extracts of subcutaneous tumors in nude mice were prepared using 0.1 M Tris-HCl (pH 7.5)/0.1% Tween 80 as a homogenization buffer. The final extracts contained 50 mg tumor tissue/μl. t-PA antigen was determined using the commercial ELISA Analyse t-PA (Biopool, Umeå, Sweden). u-PA antigen was determined with a sandwich ELISA, described in detail by Binnema et al. (6). As a standard, either purified t-PA or u-PA was used, which was standardized against the respective International Standard preparations on an activity basis using the specific activities of 500,000 IU/mg t-PA (22) and 100,000 IU/mg u-PA (WHO preparation c66/46) (48). PAI-1 and PAI-2 antigen were determined using the commercial ELISAs Tintelle Stripwell PAI-1 and Tintelle PAI-2 (Biopool). Detection limits for these assays are ~10 ng for t-PA and u-PA, 5 ng for PAI-1 and 2 ng for PAI-2 per ml.

All determinations were performed in duplicate. Serum containing non-conditioned medium was used as control.

**mRNA Analysis**

Cells were washed with PBS at 37°C and lysed in 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. RNA was isolated according to Chomczynski and Sacchi (11). The total amount of RNA isolated was determined by measuring the OD260, assuming that 1 OD260 unit is equivalent to 40 μg RNA. RNA samples were electrophoresed on a 1.2% denaturing agarose gel containing 7.5% formaldehyde and were transferred to a nylon membrane (Hybond N; Amersham International, Amersham, United Kingdom) using a VacuGene system (Pharmacia, Upplands, Sweden). Membranes were hybridized with 32P-labeled cDNA fragments in 7% SDS, 0.5 M NaHPO4, pH 7.2, 10 mM EDTA at 60°C. Blots were routinely washed with 2 x SSC, 1% SDS for 1 h at 60°C (× SSC = 0.15 M NaCl, 0.015 M sodium citrate). cDNA fragments were labeled using the random primer method (Multiprime; Amersham International), with 32P-dCTP (specific activity was ~106 cpm/μg DNA). Autoradiograms were prepared using Kodak XAR-5 films and intensifying screens at ~70°C.

**Extracellular Matrix Preparation and Degradation**

Bovine smooth muscle cells (kindly provided by Dr. G. Serti, Hamr-smith Hospital, London) were grown to confluence in DME medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 2-cm2 dishes. After the cultures had reached confluency and the formation of extracellular matrix was started, the cells were incubated for 4 d with medium containing a [3H] amino acid mixture (1 μCi/ml, Amersham International). Cells were then lysed using 0.5% Triton X-100 in PBS and the cytoskeleton was removed by 25 mM ammonium-hydroxide treatment. Unincorporated [3H] amino acids were washed from the remaining extracellular matrix using H2O (twice) and 75% ethanol. Matrices were dried and stored at ~20°C until use. Before tumor cells were seeded onto the [3H]-labeled matrices, the latter were soaked with medium for 1 h. For the extracellular matrix degradation assay 105 cells/2-cm2 dish were seeded onto the matrix in 10% FCS containing DME medium or DME medium supplemented with 100 U/ml aprotonin (as plasmin inhibitor) or specific inhibiting antibodies against t-PA (50) or u-PA (6). Antibody concentrations used were ~30 μg/ml for anti-t-PA and 300 μg/ml for anti-u-PA. Human plasminogen, purified by affinity chromatography as described previously (63), was added to the medium in all experiments to a final concentration of 0.14 μM. After 2 d, the conditioned medium was removed and the remaining matrix was degraded with 0.25% trypsin (1 h at 37°C). [3H] Amino acid release was determined and the 3H release by the tumor cells was expressed as percentage of the total amount of [3H] released (the sum of the release by the cells and by the trypsin treatment).

**u-PA Receptor Analysis**

The presence of u-PA in the cells was determined essentially according to Nielsen et al. (41). Tumor cells were grown to confluence in DME medium plus 10% FCS, detached from the culture dishes using a rubber policeman and washed twice with PBS. After a moderate acid treatment (0.05 M glycine, pH 30, 0.1 M NaCl; for 3 min at room temperature) to remove the endogenous u-PA from the receptor, cells were lysed in 0.1 M Tris-HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 100 μM aprotonin, 1 mM PMSF and centrifuged for 10 min at 10,000 g. The supernatant was stored at ~20°C. Diisopropylfluorophosphate (DFP)-treated u-PA (kindly provided by Dr. N. Behrendt, Finsen Laboratory, Copenhagen) was radiolabeled using Na125I according the iodogen procedure (Pierce Chemical Co., Rockford, IL). u-PA receptor was determined in crude cell extracts by precipitation of a sample of the cell extract (of ~25,000 cells) with 125I-labeled DFP treated u-PA, with or without addition of a 10-fold excess of unlabeled u-PA, in 20 μl PBS, 0.1% Tween 80 for 1 h at 4°C, followed by incubation with the crosslinking agent diacsianimidyl suberate (2 mM) for 15 min at room temperature and with ammonium acetate (10 mM) for 10 min at room temperature. The samples were then analyzed by SDS-PAGE and autoradiography using Kodak XAR-5 film and intensifying screens at ~70°C. Extracts of subcutaneous tumor tissue (50 mg/ml) were prepared by homogenization in 0.2 M Tris-HCl (pH 7.5). Subsequently, membrane fractions were purified by centrifugation of the extracts in an airfuge (Beckman Instruments, Fullerton, CA) at 130,000 g for 15 min. The pellet was resuspended in glycine buffer (0.05 M glycine, pH 30, 0.1 M NaCl), centrifuged.

**mRNA Quantification**

mRNA levels were determined as described by Quax et al. (48) using dot blots containing series of dilutions of in vitro transcripts of t-PA, u-PA, PAI-1 and PAI-2 as standards. These RNA transcripts were made using T7 RNA polymerase (Promega Biotec, Leiden, The Netherlands). After removal of the DNA templates with RQI DNase (Promega Biotec), the amounts of RNA transcripts synthesized were determined by measuring OD260. An internal standard β-actin was used.

**cDNA Probes**

For the hybridization experiments the following cDNA fragments were used as probes: a 1.9-kb BglII fragment of the human t-PA cDNA (61), a 1.0-kb EcoRI-PstI fragment of the human u-PA cDNA (57), a 1.2-kb PstI fragment of the human PAI-1 cDNA (58), a 1.2-kb EcoRI fragment of the human PAI-2 cDNA, kindly provided by Dr. E. K. O. Kruithof (52), and a 1.2-kb PstI fragment of a hamster β-actin cDNA, kindly provided by Dr. W. J. Quax (18).

As a probe for the u-PA receptor mRNA the p-uPAR-1 plasmid containing the complete cDNA for human u-PA (51) was used after random primer labeling and hybridization performed as described earlier (36).
fuged for 15 min at 130,000 g, resuspended in 0.1 M Tris-HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 100 U/ml aprotinin, 1 mM PMSF, and finally centrifuged for 10 min at 10,000 g. Analysis for presence of u-PAR in the supernatant was performed as described above.

**Nude Mice**

Balb/c athymic nude mice (nu/nu) were purchased from the Laboratory Breeding Research Center (Gl. Bomholtgaard, Ry, Denmark) and kept in separate rooms in cages covered with air filters under SPF conditions. Mice were used when 4-6 wk old and were sex matched.

**Tumor Cell Inoculation**

Melanoma cells were harvested from subconfluent cultures by trypsinization, washed twice with serum containing medium, suspended in PBS (1-2 x 10^6 cells/ml) and inoculated (a) subcutaneously into the lateral thoracic wall to produce tumors at the inoculation site and to produce pulmonary metastases or (b) intravenously into the lateral tail vein to produce lung colonies. 1-2 x 10^6 Tumor cells were injected in both cases. Mice were routinely checked twice a week and killed when signs of illness were noted or, when they remained healthy, 3-4 mo after inoculation autopsy was performed and kidney, spleen, liver, lymph nodes, and lungs were routinely examined for metastasis both macroscopically and microscopically. Tissue samples from subcutaneous tumors and from the lungs were snap frozen and stored at -70°C for biochemical analysis and immunohistological staining.

**Detection of Lung Metastases**

At autopsy, lungs were taken for histopathological examination, fixed in formalin, and embedded in paraffin. To avoid missing micrometastases, 4-μm hematoxylin- and eosin-stained sections from at least three different levels were examined for the presence of lung metastases (59, 60).

**Immunohistochemical Staining of u-PA**

Indirect immunoperoxidase staining was done with two well-characterized polyclonal rabbit antibodies against human u-PA of different origins (6, 32) on 4-μm frozen sections of xenograft lesions of all six melanoma cell lines. A 1-μm acetone fixation, sections were embedded as described by Nakane and Pidgeon (40). As the second antibody, swine-anti-rabbit IgG conjugated to horseradish peroxidase (Dakopatts, Denmark) was used and 3-amino-9-ethylcarbazole was used as a substrate. Harris hematoxylin (Merck, Darmstadt, Germany) was used to counterstain. As a control for specificity of the human u-PA antibodies rabbit nonimmune serum or antibodies absorbed with purified human u-PA were used (26).

**Results**

**Extracellular Matrix Degradation In Vitro**

The capacity of all six melanoma cell lines to degrade extracellular matrix was tested by culturing them on 3H-labeled extracellular matrix produced by bovine smooth muscle cells. Five of the six cell lines had the capacity to degrade the matrix (Fig. 1). Only Mel 57 showed no significant degradation of the matrix when compared to the background. In all cell lines studied, except Mel 57, matrix degradation could be inhibited almost completely by aprotinin addition (Fig. 1), indicating the involvement of trypsin-like proteases, probably plasmin, since there was a strong decrease in the matrix degradation when several cell lines were grown in the absence of plasminogen (serum free conditions without addition of plasminogen) (results not shown).

Cultivation in the presence of specific antibodies against t-PA showed a clear decrease in the matrix degradation caused by the M14, IF6, and 530 cells, but had no effect on the matrix degradation by the MV3 and BLM cells. Incubation with antibodies against u-PA showed a strong decrease in MV3- and BLM-mediated matrix degradation, but had no effect on M14-, IF6-, and 530-mediated matrix degradation. These results indicate that extracellular matrix degradation is mediated by t-PA in the M14, IF6, and 530 cells and by u-PA in MV3 and BLM cells.

**Synthesis of PA and PAI by Melanoma Cells**

To determine which types of plasminogen activators or plasminogen activator inhibitors are produced by the melanoma cell lines tested, preparations of total RNA were analyzed by Northern blot hybridizations, using cDNA probes for t-PA, u-PA, PAI-1, and PAI-2 (Fig. 2). In all the melanoma cell lines studied t-PA mRNA could be detected, while in only two cell lines, MV3 and BLM, u-PA mRNA was detected. PAI-1 and PAI-2 mRNA were also only detectable in these two latter cell lines. mRNA levels and the correspond-

![Figure 1. Matrix degradation by human melanoma cell lines.](image1)

![Figure 2. Northern blot analysis of RNA from different human melanoma cell lines.](image2)
ing protein levels were quantified in cell extracts and in conditioned media in parallel cultures (Table 1). t-PA antigen was detected in all melanoma cell lines studied and >96% of the t-PA was found in the culture medium after 24 h incubation. u-PA was only detectable in MV3 and BLM. In this case, however, a significant fraction (16 and 30%, respectively) was cell associated after 24 h incubation. PAI-1 antigen was detected in the MV3 and BLM cell lines only and >97% was found in the culture medium. The highest levels of PAI-2 were detected in MV3 and BLM, but also in the other melanoma cell lines small amounts of PAI-2 were detectable although no mRNA could be detected. In all cases PAI-2 was mainly in a cell-associated form. In general, the mRNA levels correspond well with the protein levels.

**Urokinase Cross-Linking to Cell Lysates**

Radiolabeled DFP-treated urokinase was incubated and cross-linked with lysates of all six melanoma cell lines studied. In all cell lines, a 94-kD complex was detected after SDS-PAGE and autoradiography. The intensity of the 94-kD band was similar in all the cell lines except Mel57, which showed considerably less complex formation (Fig. 3). In all cases complex formation could be prevented almost completely by addition of excess of unlabeled urokinase. These results indicate that DFP-u-PA binding is saturable and that the complexes formed have characteristics similar to u-PA/u-PAR complexes formed observed with other human cell lines (41).

**Urokinase Receptor mRNA Analysis**

RNA extracts of all melanoma cell lines were analyzed for the presence of the u-PAR mRNA using Northern blot hybridization with a specific u-PAR cDNA probe (51). In all the cell lines u-PA mRNA was detectable (Fig. 4), however the intensity of the hybridization signals varied considerably. The strongest signal was found in RNA extracts from the MV3 and BLM cells.

**Tumorigenicity and Metastatic Behavior of Melanoma Cell Lines**

To assess the tumorigenicity in nude mice, melanoma cells were injected subcutaneously. The data concerning tumor formation at the site of inoculation (tumor take) are presented.
Subcutaneous tumor development varied from 51 (M14) to 96% (MV3) of mice injected, and tumor latency ranged from 10 d (MV3) to 40 d (530). The capacity to develop lung metastases after subcutaneous inoculation is also presented in Table II, left (expressed as percentage of mice which developed subcutaneous tumors). As can be seen, two of the cell lines, IF6 and 530, did not develop metastases at all, two cell lines, M14 and Mel 57, only sporadically, and two cell lines, MV3 and BLM, very frequently. With respect to the nonmetastasizing cell lines IF6 and 530, we found in an additional experiment that even 6-7 mo after subcutaneous tumor cell inoculation or after inoculation of 10^10 tumor cells instead of the usual 1-2 \times 10^6 cells, no lung metastases could be observed. The difference in invasive character of BLM and MV3 cells compared with the less or nonmetastasizing other cell lines was also consistently observed in the subcutaneous tumors. As illustrated in Fig. 5, subcutaneous xenografts of IF6 were encapsulated by host stroma (Fig. 5 A and C), while MV3 (and BLM) cells were not (Fig. 5 B). In addition, MV3 (and BLM) cells invaded into the subdermal muscles (Fig. 5 D). As it appears from Table II, the number of lesions found in the lungs of mice that developed metastases varied strongly and both tumor cell emboli (Fig. 5 E) and invasively growing lung metastases (Fig. 5 F) were observed.

The capacity to develop experimental metastases after intravenous tumor cell inoculation was investigated in another experiment and presented in Table II, right. All melanoma cell lines, except IF6, had the capacity to colonize to the lung and form metastases with frequencies between 50 (M14) and 95% (MV3) of mice injected.

Detection of Plasminogen Activator and Inhibitor in Tumor Extracts

Antigen levels in extracts of subcutaneous tumors were determined for t-PA, u-PA and PAI-1 (Table III). Similar to the results obtained with the corresponding cultivated cells (see Table I), all tumors contained t-PA while u-PA and PAI-1 only were detected in those deriving from the MV3 and BLM cell lines.

Extracts of subcutaneous tumors were also analyzed for the occurrence of u-PA receptor by cross-linking with 121-I-labeled DFP-treated u-PA. In all tumor extracts studied, except in Mel57, 94-kD complexes could be detected, although at a low level (Fig. 6). After acid treatment of the membrane fraction of the tumor extracts (a procedure which dissociates endogenous u-PA from its receptor), a strong increase in the complex formation in the MV3 and BLM tumor extracts could be detected (Fig. 6). This indicates that receptors were present in all the tumors except Mel 57, that the levels of u-PAR in MV3 and BLM tumors were higher than in the tumors of the other cell lines, and that in these two tumors most of the urokinase receptors were occupied by u-PA, which because of a species specificity in the receptor binding (2, 21) must be of human origin.

Immunohistochemical Localization of u-PA in Tumors

To confirm the presence of u-PA in the tumors caused by the melanoma cell lines MV3 and BLM, sections of xenograft lesions were stained immunohistologically using two different u-PA-specific polyclonal antibodies. For both antibodies similar results were obtained. Fig. 7, a and c shows that BLM cells both in the subcutaneous tumor and in lung metastases derived from these tumors showed a strong, evenly distributed, u-PA staining. For MV3 a similar staining pattern was observed (data not shown). No u-PA staining was detected in the xenografts of any of the other four cell lines studied (Fig. 7 b).

Discussion

It is found that five of the six human melanoma cell lines tested were able to digest in vitro an extracellular matrix synthesized by bovine smooth muscle cells in a largely plasmin-dependent way. Degradation of the extracellular matrix was markedly inhibited by antibodies against either t-PA or u-PA. Moreover, it was blocked nearly completely by addition of aprotinin to the culture medium, which contained serum and additional plasminogen. These results suggest that plasmin is involved in the matrix degradation. As the extracellular matrices were produced by smooth muscle cells without ad-

**Table II. Tumorigenesis and Metastasis of Melanoma Cell Lines in Nude Mice**

| Cell line | Number of mice injected | Tumor take* | Lung metastasis† | Number of metastases per mouse‡ | Number of colonies per mouse§ |
|-----------|-------------------------|-------------|------------------|-------------------------------|--------------------------------|
| MV3       | 25                      | 24          | 96               | 22 (1-25)                     | 25 (3-70)                      |
| M14       | 45                      | 23          | 51               | 9                             | 25 (12-38)                     |
| IF6       | 50                      | 30          | 60               | 0                             | 0                              |
| BLM       | 60                      | 46          | 77               | 22 (3-45)                     | 11 (2-30)                      |
| 530       | 30                      | 19          | 63               | 0                             | 35 (20-57)                     |
| Mel57     | 40                      | 25          | 62               | 4 (3-24)                      | 14 (3-24)                      |

* Frequency of lung lesions in absolute numbers and as the percentage of the mice injected that gives tumor formation at the subcutaneous inoculation site.
† Frequency of lung lesions in absolute numbers and expressed as percentage of the mice injected that gives tumor formation at the subcutaneous inoculation site.
‡ Frequency of lung lesions after intravenous inoculation in absolute numbers and expressed as percentage of mice inoculated, detected microscopically as for subcutaneous inoculation.
§ Mean value of number of lesions in maximally ten randomly chosen mice with metastases or colonies counted in three lung sections from different levels.
¶ Mean value and range of lung lesions determined as for subcutaneous inoculation.

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Figure 5. Morphological aspect of subcutaneous tumors after inoculation of IF6 cells (A and C) and MV3 cells (B and D) and of the metastases in lungs of mice subcutaneously inoculated with MV3 cells (E and F). In the lung sections both tumor cell emboli (E) and invasively growing metastases (F) were found. All sections were stained with haematoxylin and eosin. Bar, 1 μm.

Table III. PA and PAI Protein Levels in Subcutaneous Xenograft Lesions

| Tumors derived from cell line | t-PA   | u-PA | PAI-1 |
|------------------------------|--------|------|-------|
| MV3                          | 0.060  | 0.91 | 5.3   |
| M14                          | 0.042  | -    | -     |
| IF6                          | 0.32   | -    | -     |
| BLM                          | 0.072  | 0.28 | 0.56  |
| 530                          | 0.29   | -    | -     |
| Mel57                        | 0.036  | -    | -     |

Protein levels of plasminogen activators and inhibitor type 1 in extracts of subcutaneous tumors derived from human melanoma cell lines in nude mice (dashes indicate levels below detection limits, 0.02 and 0.01 ng/mg for u-PA and PAI-1, respectively).

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Addition of ascorbic acid to the culture medium they contain little or no collagen (10, 29, 30). The matrix degradation was mediated virtually only by t-PA in three of the cell lines (M14, IF6, and 530) while in two cell lines (BLM and MV3) it was mediated virtually only by u-PA (Fig. 1). Very little, although significant matrix degradation was observed with Mel57 cells. This matrix degradation could not be inhibited by antibodies to u-PA or t-PA and could only partly (50%) be inhibited by aprotinin. The finding that u-PA can mediate degradation of extracellular matrix in this system is in agreement with a previous report by Cajot et al. (10), in which mouse L-cells transformed with the human u-PA gene were studied.

All melanoma cell lines studied produced large quantities of t-PA mRNA and antigen, which was mainly secreted into
Figure 6. Analysis for u-PA receptor in extracts of subcutaneous tumors derived from human melanoma cell lines (A) or isolated, acid treated, membrane fractions (B) were incubated with DFP u-PA. After cross-linking with disuccinimidylsuberate, samples were analyzed using SDS-PAGE and visualized using autoradiography. Lysates of U937 cells were used as a positive control.

An interesting observation is that although all cell lines produce t-PA, matrix degradation caused by the MV3 and BLM cells could not be inhibited by anti-t-PA while anti-u-PA inhibited the matrix degradation markedly. A possible explanation might be inhibition of t-PA by PAI-1, in contrast u-PA in the medium is probably unaffected by PAI-1 because it is mainly present in its proenzyme form (I). Receptor-bound u-PA can be inhibited by PAI-1 (13) but the cell surface pathway of u-PA catalyzed plasminogen activation may still be efficient in the degradation of the extracellular matrix due to (a) a different location of u-PA and PAI-1, as it has been observed in some cell types (43); (b) the enhancement of plasmin formation caused by concomitant binding or pro-u-PA and plasminogen to cell surfaces (19, 54); (c) a protection of surface-bound plasmin from its serum inhibitor α2-antiplasmin (44, 54); and (d) high local concentrations of plasmin at the interphase between the cells and the matrix.

All the human melanoma cell lines could to some extent
produce subcutaneous tumors at the site of inoculation. Since human melanoma cell lines were used in mice it was possible to study the expression of t-PA, u-PA, and PAI-1 by the tumor and the metastases in vivo in the mouse tissue, using species specific antibodies. The expression of human t-PA, u-PA, and PAI-1 in these tumors in vivo corresponded well with the expression in vitro. t-PA was found in extracts of all the tumors, while u-PA and PAI-1 was found only in the MV3 and BLM tumors. The presence of u-PA in the MV3 and BLM tumors and its absence in the other tumors was confirmed by the immunohistochemical studies. No mouse u-PA could be detected in the tumors (not shown). In extracts of all the subcutaneous tumors, except the Mel57-derived tumor, u-PAR was detectable. After mild acid treatment, u-PA binding to lysates of the MV3 and BLM tumors increased dramatically, indicating that in these two cases the receptor was already largely occupied by u-PA produced by the human tumor cells, because the human urokinase receptor can not efficiently bind mouse u-PA (2, 21).

Of the six cell lines only MV3 and BLM frequently developed lung metastases. These two cell lines were also the only ones having u-PA mediated matrix degradation in vitro and u-PA and PAI-1 production in vitro and in vivo. The melanoma cell lines that only produce t-PA did not, or only sporadically, develop lung metastases after subcutaneous inoculation. The cells forming metastasis sporadically, Mel57 and M14, might use other proteolytic enzymes. Matrix degradation experiments with Mel57 reveal no inhibition by antibodies against t-PA and u-PA and only a very limited effect of aprotinin, suggesting the involvement of non-serine proteases. These observations suggest a correlation between u-PA production, PAI-1 production and frequent spontaneous metastasis after subcutaneous inoculation. Statistical analysis reveals that there is only a 0.5–7% probability that this correlation is based on chance, depending on the fact whether u-PA and PAI-1 production are completely independent or strictly coupled.

All the human melanoma cell lines, except IF6, caused lung colonies in at least 49% of the mice when inoculated intravenously in the tail vein (Table II B). The differences in metastasis formation after subcutaneous versus intravenous inoculation suggests that u-PA and/or PAI are involved especially in the early steps in the metastatic cascade and not in the lodgement of melanoma cells in the lungs. This conclusion is in contrast to previous reports in which a role of plasminogen activators in formation of lung colonies after intravenous inoculation of tumor cells was suggested in studies with transfection of t-PA and u-PA genes (3) or with inhibition of u-PA by preincubation with anti-catalytic u-PA antibodies (27).

Our findings suggest that the ability to metastasize after subcutaneous inoculation is not only related to u-PA production but possibly also to the production of PAI-1 and PAI-2. In some cell types receptor-bound pro-u-PA and u-PA is localized to cell–cell and focal–cell substratum contact sites (28, 45, 46, 54), and it has been proposed that a selective activation of pro-u-PA on some of these contact sites may lead to a directional proteolysis involved in cell migration (46, 17). A similar role might also be played by t-PA when it occurs in a surface bound form (7). PAI-1 and PAI-2 are effective inhibitors of u-PA even when it is receptor bound (4, 13, 14, 20, 47) and it is possible that the inhibitors are required for a finely tuned regulation of the surface plasminogen activation during migration of the cancer cells in the invasive process.

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