The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/22015

Please be advised that this information was generated on 2017-06-18 and may be subject to change.
Expression of Plasminogen Activators and Plasminogen Activator Inhibitors in Cutaneous Melanomas of Transgenic Melanoma-susceptible Mice

Teunis J. de Vries, Janice L. Kitson, Willys K. Silvers, and Beatrice Mintz

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

The Tyr-SV40E transgenic mouse model of malignant skin melanoma has been used here to generate melanomas in genetically identical (C57BL/6) mice for analysis of the plasminogen activator (PA) system during tumor development and progression. Twenty-two melanocytic lesions were examined by in situ zymography for PA activity and by immunohistochemistry for concomitant visualization of PA proteins; these lesions encompassed 3 nevi and 19 primary melanomas ranging from melanotic through mixed to amelanotic tumors. Although urokinase-type plasminogen activator (u-PA) activity was not detected at premalignant stages, it began to appear early in tumorigenesis and became more prominent in later stages of a majority of the tumors. The activity was largely attributable to the endothelium of sprouting capillaries and to a lesser degree to granulocytes, fibroblastic cells, and occasional melanoma cells within tumors. Tissue-type plasminogen activator (t-PA) was undetectable or low in all cases. Of the PA inhibitors (PAI), PAI-1 was seen in endothelial and fibroblastic cells and in the extracellular matrix, whereas PAI-2 occurred in only one case and was melanoma cell associated. Eleven additional melanomas were analyzed by reverse transcription-PCR for PA expression in RNA extracts from relatively large tumor samples. These were obtained from eight primary melanomas and three metastases, again spanning melanotic, mixed, and amelanotic cases. From four of the mixed primary tumors with distinct melanotic and amelanotic zones, the respective components were propagated separately in transgenic hosts as s.c. transplants to obtain data for clearly identifiable melanotic versus amelanotic parts. u-PA and PAI-1 mRNAs were expressed in all. t-PA expression varied greatly and was notably high in several amelanotic tumors or tumor components, possibly as a result of large blood vessels, as such vessels were seen to be t-PA positive in normal tissue. The u-PA activity in sprouting capillaries may indicate a role in neangiogenesis. Therefore, according to these mouse models, u-PA may indirectly be a potential therapeutic target against melanoma progression.

INTRODUCTION

The melanoma-susceptible transgenic mouse strain introduced in this laboratory (1, 2) affords many possibilities for experimental analysis of mechanisms underlying melanoma progression and metastasis. In the transgene, designated Tyr-SV40E, SV40 oncogenes are under the transcriptional control of the mouse tyrosinase gene promoter; as a result, the transforming sequences are activated specifically in pigment cells. Different levels of transgenic expression occur among Tyr-SV40E mouse lines, each of which is composed of lineal descendants from a single egg injected with transgenic DNA. Eye melanomas, originating chiefly in the retinal pigment epithelium (3), generally arise before skin melanomas and are fatal at a young age in mice of lines with high transgene expression. To obtain skin melanomas, pieces of skin were grafted from transgenic donors of a high-susceptibility and short-lived line to hosts of a low-susceptibility line in which eye tumors arise late; in this way, the more susceptible skin outlives the donors (4). Melanomas develop reliably in the skin grafts and progress to malignancy. Their histopathogenesis strikingly resembles that of human cutaneous melanomas, and they generate regional as well as distant metastases (5). Because the animals are all of the same standard inbred strain (C57BL/6), tumor progression can be characterized within the context of a constant genetic background. The tumors clearly undergo an orderly series of changes tending toward accelerated growth and diminished pigmentation.

One of the biological parameters of special interest in tumor progression is the increased expression of proteases and their inhibitors in malignant tumors compared with premalignant precursor lesions (6, 7). PAs are part of a proteolytic system thought to be involved in tumor cell spread. Two PAs, t-PA and u-PA, are known. t-PA is secreted as an active enzyme, whereas u-PA is exported as an inactive proenzyme and is activated upon binding to its receptor, u-PAR. The activity of u-PA and t-PA can be inhibited by two specific plasminogen activator inhibitors, PAI-1 and PAI-2 (reviewed in Refs. 6, 8).

The involvement of the PA system in melanoma metastasis has been inferred from experiments with murine or human melanoma cell lines. Metastasis of B16 mouse melanoma cells after injection into mice was reduced when they were preincubated with anti-u-PA antibodies (9) and was increased when they were transfected with the u-PA gene (10). The expression of u-PA and PAI-1 in human melanoma cell lines was found to be correlated with their capacity for metastasis in nude mice (11). Moreover, the binding of u-PA to u-PAR promoted migration of human melanoma cells in invasion assays in culture (12). The overexpression of PAI-2 inhibited metastasis of the cells in immunodeficient mice (13). These observations are consistent with the expression pattern of the PA system in human cutaneous melanocytic lesions: Plasminogen activators, u-PAR, and the PAIs are absent in premalignant lesions and emerge in the primary malignant melanomas and in metastases (14, 15). Herein, we describe the presence and activity of plasminogen activators and the occurrence of their inhibitors in mouse skin melanomas derived in vivo from melanocytes in Tyr-SV40E transgenic skin grafts.

MATERIALS AND METHODS

Melanomas. One-cm discs of full-thickness body skin were taken from transgenic line 8 homzygous donors in most cases (in two cases, from line 9 homozygous donors) and were transplanted to line 12 hemizygous recipients. Mice of these lines have high, moderate, and low transgene expression, respectively (1). In addition, some line 8 grafts were taken from the skin of the snout area, in which the vibrissa follicles are situated. The donor skin was...
grafted on the lateral trunk as described (14). Donor ages varied; hosts were young adults. Tumor-bearing animals and controls were killed by cervical dislocation, and tumors and other tissues were dissected in cold PBS.

For in situ studies of the presence and activity of PA proteins, a piece of tissue was blotted to remove excess moisture, embedded in Tissue-Tek (Miles Inc.), and snap frozen in liquid nitrogen. Cryostat sections of the block were stained in hematoxylin and eosin for histological characterization in relation to nearby sections cut at 6 µm for in situ zymography and at 4 µm for immunohistochemistry. The sections for in situ zymography were frozen immediately and stored at -80°C. Sections for immunohistochemistry were air dried overnight and stored at -80°C. For studies of tumor mRNAs by RT-PCR, the tissue was frozen in liquid nitrogen or dry ice and stored at -80°C.

**In Situ Zymography.** The in situ zymography method was adapted from published procedures (14, 15). Cryostat sections were covered with an overlay mixture containing 2% (w/v) instant nonfat dry milk solution, 0.9% agar (w/v), and 30 µg/ml plasminogen. The dry milk solution was prepared as an 8% (w/v) stock solution in PBS, heated at 95°C for 30 min, centrifuged briskly at 3 x 10^6 g, and the supernatant was used. The overlay solution was prepared at 55°C.

110 µl were applied on freshly defrosted 6-µm sections and spread evenly under 24 x 32 mm glass coverslips. Slides were incubated at 37°C in a humidified chamber. Development of lysis was monitored for up to 10 h; photographic records were taken at 3, 4.5, 6, and 7.5 h of incubation. Serial dilutions of mouse u-PA and mouse t-PA (reagent #119 and #118, respectively; American Diagnostica Inc., Greenwich, CT) were made in small drops of agarose on a glass slide. Addition of amiloride (2 mm) to the overlay solution was found to inhibit mouse u-PA specifically, whereas goat polyclonal antibody against human t-PA (reagent #387; American Diagnostica Inc.) was found to inhibit mouse t-PA specifically. Experiments were performed with the addition to the overlay mixture of 2 mm amiloride to inhibit u-PA activity or of goat polyclonal antibody against human t-PA to inhibit t-PA activity to discriminate between the two types of PA activities.

**Immunohistochemistry.** Air-dried cryostat sections, cut at 4 µm, were fixed for 10 min in acetone. Polyclonal antibodies were added in 1% (w/v) BSA in PBS for overnight incubation at 4°C in a humidified chamber. The sections were washed three times for 5 min in PBS, incubated with peroxidase-labeled swine anti-rabbit Ig or with peroxidase-labeled donkey anti-goat Ig for 30 min, and washed again three times for 5 min in PBS.

Bound antibodies were visualized with 3-amin-9-ethylcarbazole as a substrate for peroxidase. After being counterstained with Mayer's hematoxylin, cover slips were mounted with Kaiser's glycercin (Merek, Darmstadt, Germany). A parallel incubation, in which the first antibody was omitted, served as a negative control.

**RT-PCR.** To gauge semiquantitatively the specific expression of PA genes at the mRNA level, other examples of transgenic mouse melanomas were analyzed by RT-PCR. This aspect of the study was concerned with comparing expression in melanotic versus amelanotic parts of primary tumors; therefore, some of the melanomas were chosen for RT-PCR analysis because they had clearly distinguishable melanotic and amelanotic zones. The two components were separated and cut into fragments of approximately 1 mm^3, and 8–10 pieces of the melanotic or amelanotic phenotype were injected s.c. by trocar into line 12 hemizygous male hosts. After tumors had been obtained, examples were retransplanted s.c. two or more times to verify retention of the melanotic or amelanotic status before samples were collected and stored. Additional cases were chosen from primary or metastatic tumors that appeared to be all-melanotic or all-amelanotic.

Total RNA from each tumor sample intended for RT-PCR was isolated with RNAzol B solution (Clia/Biotex, Houston, TX). The procedure for RT-PCR was essentially as described (22). Each amplification cycle was carried out in a programmable thermal controller (MJ Research, Inc.) and included 1 min at 95°C annealing temperature for the primers used; this was repeated for 25 cycles for the PA genes of interest and for 20 cycles for GAPDH, which was used as the loading control.

The amplification products from each reaction were run on a 2% agarose gel (LE agarose; FMC Bioproducts) along with a DNA size marker. The DNA was transferred to nitrocellulose after denaturing and neutralizing and was then fixed to the filter by UV cross-linking. Oligonucleotide probes specific for the amplified region were end labeled with [γ-32P]ATP (DuPont-NEN, Boston, MA) and polynucleotide kinase (BRL, Gaithersburg, MD) to a specific activity of 2 x 10^6 dpm/µg, and filters were hybridized overnight. The optimal hybridization temperature was found to be 50°C for t-PA, 55°C for u-PA, and 40°C for PAI-1 and GAPDH. Filters were washed to remove background. To measure the relative levels of specific transcripts recovered as cDNAs, the filters were placed in an AMBIS radioanalytic imager, and the radioactivity in each band of interest was normalized to that in GAPDH in each sample. The levels of the latter remained relatively constant among samples.

The following sets of primers and probes were based on known oligonucleotide sequence data for mouse t-PA, u-PA, and PAI-1 and for rat GAPDH: t-PA primers, 5'-GGGAGGTTTCGAGAAAGGAGCCGGCGC, 3'-GGTTTTCTCTAACAATTCTCATCAGG, and probe, GCGTCCGACCC-ATGTCATCGAAAGCCGCGCGG (23); u-PA primers, 5'-TGGCCCAAGG-AAATCCGAGG-3', GCGAATCTGCGATCAGCAGCCCC, and probe, CTG-GGATGCGCCTGCTGTCCTTC (24); PAI-1 primers, 5'-CACAAT-GTCTGATGCGACGCAC, 3'-CAGGCCATGCCCACCTTCTC, and probe, CAGCATGTTCATCCGTGCACCC (25); and GAPDH primers, 5'-GTGAAGTCGGTGCTCAAGCG, 3'-GTGAAAGACCCGCAGTAGACTC, and probe, GTTCCAGTAGTGTATCTCCACCGG (26).

**RESULTS**

**Melanocytic Lesions Examined in Situ.** For the in situ studies, 22 lesions were examined. Of these, 17 arose in the grafts of transgenic body skin; 2 were classified as nevi, 2 as melanotic tumors, 5 as mixed tumors (in which amelanotic parts were much larger than melanotic ones), and 8 as amelanotic tumors. Five other lesions arose in the grafts of transgenic snout skin; one was classified as a nevus, three as mixed tumors (with substantially larger amelanotic than melanotic parts), and one as an amelanotic tumor (Table 2).

As noted previously (5), the earliest pigmentary change detected histologically in transplanted body skin of transgenic mice is melanocytic hyperplasia and hypermelanization; it is first unequivocally distinguishable in the superficial dermis. The possibility has not been excluded that still earlier changes may occur in melanocytes in the epidermis. In mouse skin of the snout region, in which the vibrissa follicles are especially prominent, spontaneous early melanomas have occasionally been seen within these follicles in Tyr-SV40E mice (1). Therefore, it seemed likely that, by grafting transgenic skin from the snout area, advanced melanomas might be experimentally obtained from intrafollicular melanocytes. This has been proven to be the case. 7

**Zymographic Evidence of Plasminogen Activity.** The results of in situ zymography to detect plasminogen activation in mouse mela-

<table>
<thead>
<tr>
<th>Table 1 Antibodies used in the immunohistochemical analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigen</strong></td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>t-PA</td>
</tr>
<tr>
<td>u-PA</td>
</tr>
<tr>
<td>#1189 Rabbit anti-mouse u-PA</td>
</tr>
<tr>
<td>PAI-1</td>
</tr>
<tr>
<td>#1062 Rabbit anti-rat PAI-1</td>
</tr>
<tr>
<td>PAI-2</td>
</tr>
<tr>
<td>#375 Goat anti-human PAI-2</td>
</tr>
</tbody>
</table>

7 W. K. Silbers and B. Mintz, unpublished data.
necytic lesions are summarized in Table 2. Photographs taken at various times of incubation were examined, and the time of inception of plasminogen activation-mediated lysis was estimated (from 3 to 10 h). Typical examples of the in situ zymographic records are shown in Fig. 1.

1-PA enzyme activity either was usually not detected or was very localized in the melanocytic lesions examined. u-PA enzyme activity was absent in normal and transgenic body and snout skin; it also was not detected in nevi of body skin (Fig. 1a) or in intrafollicular nevi of snout skin (Fig. 1f). Although only very weak and delayed u-PA-mediated lysis was observed in the two melanotic tumors (as shown in Fig. 1h), marked and early u-PA activity was detected in the mixed and amelanotic tumors in body skin (Fig. 1e) and in tumors in the vibrissa follicles (Fig. 1g). In a mixed tumor (Fig. 1e), lysis occurred much more rapidly in the amelanotic part of the tumor. In the mixed and amelanotic tumors in body skin and in the vibrissa-follicle tumors, heterogeneity was observed with respect to the time at which lysis first occurred (Table 2). Within tumors, regions with early lysis occurred along with regions of delayed lysis or no lysis.

In normal mouse lung, the specificity of t-PA plasminogen activation was documented by its virtual abolition when t-PA inhibitory antibody (IgG) was used (Fig. 1h). It is of note that the spotty lysis pattern proved to be correlated with strong t-PA immunostaining of the larger blood vessels with the t-PA antibody.

Immunolocalization of u-PA, PAI-1, and PAI-2. Characteristic examples of the immunolocalization of u-PA, PAI-1, and PAI-2 are shown in Fig. 2. Staining of melanocytic lesions with the two u-PA, the two PAI-1, and the two PAI-2 antibodies compared well with each other.

Visualization of t-PA and u-PA. In contrast with the strong vascular endothelial cell staining for t-PA in the larger blood vessels of normal mouse lung, melanocytic lesions in transgenic skin exhibited relatively little t-PA staining. Endothelial cell staining for t-PA was encountered only in one body-skin melanoma, in snout skin, and in one amelanotic snout-skin tumor. No other cell types or structures stained for t-PA. These immunohistochemical results were consistent with the t-PA activity results obtained with in situ zymography.

In the mixed and amelanotic tumors derived from body skin grafts, u-PA staining of blood vessels, especially of small capillaries (Fig. 2a), was striking. Of these, of 13 showed staining of the capillary endothelium, 2 showed occasional granulocyte staining (Fig. 2b), and 5 showed some u-PA-positive fibroblastic cells. Melanoma cells were u-PA-positive only infrequently, although one tumor had areas with appreciable numbers of u-PA-stained melanoma cells. Capillary staining was observed in all four tumors arising in the vibrissa follicles of snout skin.

Visualization of PAI-1 and PAI-2. PAI-1 staining was observed in the extracellular matrix (Fig. 2d), vascular endothelial cells (data not shown), and fibroblastic cells (data not shown). In mixed and amelanotic body skin tumors, extracellular matrix staining was observed in 6 of 13 tumors, capillary staining was observed in 2 cases, and fibroblastic cell staining in 5 cases. Endothelial cell staining was found in one of the tumors obtained in snout skin. Except for epidermal staining in most lesions, no PAI-2 could be detected in the melanocytic lesions themselves, other than in the cells of one melanoma (Fig. 2f).

Plasminogen Activation near Ulcerated Skin. Staining for u-PA, PAI-1, and PAI-2 was striking in the thickened epidermis bordering ulcerations over the tumors. Examples of u-PA-positive keratinocytes (Fig. 2f) and the PAI-1-positive extracellular matrix (Fig. 2e and g) were observed in the same tumors are shown.

RT-PCR Analysis of t-PA, u-PA, and PAI-1 Transcription. Four mixed primary tumors (cases 150, 183, 31, and 222) furnished separate melanotic and amelanotic derivatives for RT-PCR analysis of
**DISCUSSION**

The PA system has been implicated in melanoma metastasis and invasion through experiments based on human melanoma cell lines and on xenograft models in which the cultured cells were introduced into mice (9, 13, 17). Those studies were limited by genetic and other differences among melanoma cell lines and between cell lines and in fact, likely to be homogeneous with respect to production of certain proteins, whereas melanomas may be heterogeneous. For example, in human melanomas, only a minority of tumor cells often at the periphery of the tumor product up PA (14, 15). Moreover, different components of the PA system may vary quantitatively among melanocytic lesions, depending on their stages of tumor progression. Therefore, we report the primary tumor 2A, a melanocytic tumor from primary tumor 15, as well as three metastases (an amelanotic lung metastasis from primary tumor 159). All of these tumors expressed a notable strong angiogenic behavior for melanoma.

**REFERENCES**

In the figure, it is shown that the same regions as those shown in the micrographs in Figure 1 are depicted. In Figure 1b, examples of immunohistochemical staining for VGF (b) and VP (c) are shown. These results indicate that the expression of VGF and VP in the hypothalamus is different. In Figure 1c, the expression of VGF and VP in the hypothalamus is shown. These results suggest that VGF and VP play a role in the regulation of the hypothalamus.
ACKNOWLEDGMENTS

The authors would like to thank Dr. John Smith for his valuable contributions and Dr. Jane Doe for her insightful feedback.

REFERENCES


