Comparative Decreases in Tyrosinase, TRP-1, TRP-2, and Pmel 17/Silver Antigenic Proteins from Melanotic to Amelanotic Stages of Syngeneic Mouse Cutaneous Melanomas and Metastases

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ABSTRACT

Malignant cutaneous melanomas and metastases were taken directly from in situ lesions of genetically identical (C57BL/6 strain) Tyr-SV40E transgenic mice, and samples were analyzed by Western immunoblotting with antisera specific for the COOH terminus of each of four melanocytic proteins. These were tyrosinase, TRP-1, TRP-2, and Pmel 17/silver. Of the 13 melanomas examined, there were 5 melanotic primary tumors, 5 amelanotic primary tumors, and 3 amelanotic metastases. The melanocytic tumors expressed all of the markers to some extent. In contrast, the amelanotic tumors lacked detectable levels of one, two, or three of the proteins, except for an apparently amelanotic tumor sample in which all were expressed, but in which some melanocytic cells were likely to have been present. Thus, despite some variability, there is clearly a downward trend in the presence of these proteins as the tumors become amelanotic, a pigmentary change associated with ongoing malignant progression. In the amelanotic tumors, tyrosinase was most often deficient, whereas TRP-2 was most often persistently expressed. These results, obtained from melanomas of syngeneic origin, indicate that tumors in the relatively early stages of malignancy might be more responsive than later-stage tumors to immunotherapy involving an ensemble of antigenic peptides of the tested gene products. Moreover, TRP-2 peptides may be especially useful for therapeutic intervention at the later stages.

INTRODUCTION

The normal synthesis and deposition of melanin is a highly regulated process restricted to melanocytes and retinal pigment epithelium. The participation of many pigment genes is required. Proteins encoded by a number of the genes have been identified in the melanin biosynthesis pathway or localized to the melanosome, the organelle in which melanization occurs. Included are tyrosinase, which controls the rate-limiting step in melanogenesis, the allied gene products TRP4-1 and TRP-2, and Pmel 17/silver, which may contribute to a melanosomal matrix for melanin deposition. (Pmel 17 and silver both refer to the product of the mouse locus originally designated the silver locus; gp100 refers to the product of the homologous human locus.)

These and other melanocytic lineage proteins or their peptide derivatives are potential targets for immunotherapy of melanoma. Recent work by a number of groups supports this possibility. For example, CTLs were isolated from the tumors of melanoma patients and tested for responsiveness to cultured nonmelanoma cells transfected with cDNAs and expressing major histocompatibility molecules. Peptides derived from tyrosinase (1-4), TRP-1 (5), TRP-2 (6), and gp100 (4, 7-10) were identified as antigens recognized by the melanoma-reactive T cells and thus as possible candidates for protein- or peptide-directed immunotherapy. However, the ability of an antigen to mediate an effective antitumor immune response requires expression of that antigen by the tumor cells in situ. Human melanomas have, therefore, been examined for expression of mRNAs of tyrosinase, TRP-1, TRP-2, and gp100, and/or the melanoma-associated antigens themselves (11-15). Results differ from one study to another. All reveal discordant expression of the markers, especially in late-stage tumors, and have led to proposals for pretreatment typing of lesions as a basis for determining the most suitable vaccine for a given patient.

Evaluation of human melanomas is complicated by the genetic heterogeneity of the patient population. Cell lines from tumors have the further difficulty that they are likely to have undergone selection. We have, therefore, undertaken the characterization of melanomas arising in genetically identical Tyr-SV40E (C57BL/6 inbred strain) transgenic mice (16, 17). These tumors, at various stages of progression, are being analyzed at the mRNA (18, 19) and protein (20) levels for expression of specific melanogenic mouse genes for which human homologues are known. The animals provide many experimental possibilities and are useful models for therapeutic intervention.

In a previous report (20), we compared levels of the mouse tyrosinase, TRP-1, TRP-2, and silver proteins in melanotic versus amelanotic tumor components removed from zonal primary melanomas and metastases of Tyr-SV40E transgenic mice and transplanted s.c. in transgenic hosts. Here we describe the representation of the same antigenic proteins in original (nontransplanted) melanomas at progressive stages. The results reveal a variable but relatively orderly succession of changes in the presence of these proteins.

MATERIALS AND METHODS

Melanomas. The melanomas arose in grafted skin of melanoma-susceptible Tyr-SV40E transgenic mice (16) of the C57BL/6 inbred strain, after grafting a small disc of full-thickness body skin from line 8 hemizygous (high-susceptibility) donors to line 12 hemizygous (low-susceptibility) hosts (17). The transgene is expressed in melanocytic-lineage cells due to a controlling sequence of the tyrosinase gene. The oncogenic (SV40) sequence acts as a tumor-initiating stimulus; factors associated with wound healing supply a tumor-promotional stimulus. Malignant melanomas then develop in the grafted skin and metastasize into organs of the hosts. The graft-donor provenance of the malignant cells is evident from the donor-type Southern blot pattern of the integrated transgene (17). The tumors are at first melanotic, and in line 8 skin, they often progress to an apparently amelanotic or hypomelanotic state. Metastases may also be melanotic, amelanotic, or mixed. Tumor pieces were frozen in dry ice and stored at -70°C.

Thirteen melanomas were analyzed here. Among them were 10 primary cutaneous tumors (5 melanotic and 5 apparently all or largely amelanotic) ranging in size from approximately 1200–3000 mm², and amelanotic metastases originating from three of the primary tumors. One of the analyzed metastases was in lung and two were in liver. (Additional macrometastases in lymph nodes and lungs from three of the other primary tumors were not analyzed.)

Western Immunoblot Analysis. Melanoma samples (or a pellet of control melanocytes cultured from C57BL/6 nontransgenic mouse skin) were extracted by...
homogenization in a Dounce homogenizer on ice, and protein concentration was determined as described (20). Per sample assayed, 20 μg of protein were denatured by boiling for 90 s in SDS and 2-mercaptoethanol to 2% final concentration and electrophoresed in SDS/7.5% polyacrylamide gel; the gel was then subjected to immunoblotting as described (21). Rabbit antipeptide antisera, each directed against the COOH terminus of the protein encoded by a murine gene, were gifts from Vincent J. Hearing of the NIH. These were: aPEP7, against tyrosinase; aPEP1, against TRP-1; aPEP8, against TRP-2; and aPEP13, against the silver or Pmel 17 protein (22–24). After immunoblotting, autoradiograms exposed for identical periods were processed by image capture with an Alpha Innotech IS-1000 digital imaging system.

RESULTS AND DISCUSSION

The immunoblot results in Figs. 1–4 clearly show that the five melanotic primary melanomas were positive to some extent for all melanogenic proteins examined, whereas only one of the five amelanotic primary melanomas (456P-amel) was positive for all of the marker proteins. Tumor 456P-amel had, in fact, been recorded at autopsy as appearing largely amelanotic, with occasional small melanized areas; thus, the “amelanotic” sample chosen for analysis may well have contained some melanotic cells. Of the other primary tumors described here as amelanotic, none had any obviously melanized components; each of those tumors lacked any detectable level of one, two, or three of the tested proteins. The three amelanotic metastases had variable protein levels, often similar to the cutaneous tumor of origin. Taken together, the results point to a gradual downward trend in expression of these proteins during the transition from a melanotic to an amelanotic state. This pigmentary change is characterized with increased mitotic activity and ongoing malignant progression in human melanomas (25) and also in our mouse melanomas. It should nevertheless be emphasized that a cutaneous melanoma is often still melanotic when metastasis occurs, in both humans and mice. An example in the mice examined here is tumor 351P-mel, which gave rise to (unanalyzed) melanotic metastases in lymph nodes and lungs.

Fig. 1. Western immunoblot analysis of tyrosinase protein levels in 10 primary (P) melanotic (mel) or amelanotic (amel) cutaneous melanomas from Tyr-SV40E transgenic (C57BL/6 strain) mice and in a metastasis (M) from each of three of the cases. Metastases were in lung (Lu: 753M) and liver (Li: 455M and 710M). The blots in the upper panels are shown with the densitometric readings in the corresponding histograms below. Left, size markers of known Mr. Melanotic tumors (I), amelanotic tumors (II), control (Co) value obtained from melanotic melanocytes cultured from nontransgenic C57BL/6 skin. Units of measurement in the histograms are density values x 10⁻³; results for tumors should be compared with the level of the control in the same blot.

Fig. 2. Western immunoblot analysis of TRP-1 in the same panels of transgenic mouse melanomas as in Fig. 1.

Fig. 3. Western immunoblot analysis of levels of TRP-2 in the same panels of transgenic mouse melanomas as in Fig. 1. (Only the specific band at Mr, 75,000 was analyzed; the lower band is also present in nonmelanocytic tissues.)

Fig. 4. Western immunoblot analysis of levels of the Pmel 17/silver protein in the same panels of transgenic mouse melanomas as in Fig. 1.

5W. K. Silversand B. Mintz, unpublished data.
The data also demonstrate that tyrosinase was the protein most often absent or low in the amelanotic tumors (Fig. 1), whereas TRP-2 was most often present and was also most highly represented relative to controls in the same blots (Fig. 3). TRP-1 and silver proteins were intermediate, with TRP-1 represented more often than silver. Only one tumor (750P-amel) lacked any detectable TRP-2 protein. This tumor was also negative for the tyrosinase and silver proteins and had only a low level of TRP-1; it was, therefore, the least differentiated of intermediate, with TRP-1 represented more often than silver. Only to controls in the same blots (Fig. 3). TRP-1 and silver proteins were

established murine B16 melanoma cell line, after being engineered to

the changing pigmentary phenotype of the tumors. The initial loss of

concomitant with malignant progression. Differences in persistence of

was most often present and was also most highly represented relative

possible routes would be effective antigens, or could be experimentally

and tyrosinase genes: a study in an unselected group of HLA-A2.1-positive


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