

IN SITU ANALYSIS OF ANTIGENS ON MALIGNANT AND BENIGN CELLS OF THE MELANOCYTE LINEAGE

Differential Expression of Two Surface Molecules, gp75 and p89

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The hypothesis that cells become malignant through a multistep process has been developed from studies on chemically induced tumors (1) and is supported by the demonstration that multiple oncogenes are required to induce malignancy in vitro (2). Malignant melanoma is a spontaneous human tumor in which clinical and histopathological criteria can be used to define a series of proliferative lesions that apparently constitute different steps in the evolution from melanocyte to malignant melanoma (3). These alterations in growth pattern occur sequentially and appear focally within the respective preceding lesion. It would be of great value to know whether discrete successive steps in the development of melanoma can be correlated with changes in the expression of distinct molecules. Since skin lesions with altered growth of pigment cells can be easily recognized and removed, one approach to this question is to use monoclonal antibodies (mAb)¹ (4) as probes on histological sections of such lesions. In the study reported here, a series of benign and malignant melanocytic lesions were examined using a panel of mAbs reactive with malignant melanoma. Two antigens were identified that appear to be differentially expressed on malignant melanoma and their nonmalignant precursor lesions.

Materials and Methods

Tissues and Cells. Cells were isolated from lymph node metastases of malignant melanomas by mincing the tissues in phosphate-buffered saline (PBS), pH 7.2. The released cells were washed twice with PBS and stored at -70°C in RPMI 1640 containing 20% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO).

Peripheral blood mononuclear cells (PMC) were isolated from peripheral blood by Ficoll-Hypaque centrifugation and phytohemagglutinin (PHA) blasts examined after 5 d of stimulation. Red cells were prepared from the Ficoll pellet and granulocytes were obtained by lysing the red cells with 0.15 M NH_4Cl containing 1 mM EDTA. Tissues were frozen immediately after surgical removal in isopentane precooled in liquid nitrogen

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¹ *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HAT, hypoxanthine aminopterin thymidine; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMC, peripheral mononuclear cells; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

or directly in liquid nitrogen, and stored at -70°C . The melanoma cell line Mel Ei was established in our laboratory (5). All cells were maintained in RPMI 1640 containing 10% FCS and 1 mM sodium pyruvate.

Chemicals. Where not otherwise stated, chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Antibodies. mAb 691-13-17 (IgG1) was obtained from M. Herlyn, The Wistar Institute, Philadelphia (6), and mAb DA6.231 (IgG1) was obtained from K. Guy, MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh (7). Both antibodies are directed against nonpolymorphic determinants on human Ia-like antigens. The hybridomas producing mAb BBM.1 (IgG2b) specific for β_2 -microglobulin (8) and mAb 5E9 (IgG1) that recognizes the transferrin receptor (9) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. mAb 96.5 (IgG2a, obtained from I. Hellström, Fred Hutchinson Cancer Research Center, Seattle) defines a 97 kD glycoprotein that was shown by iron-binding studies and protein sequence analysis to be related to transferrin (10). MPC11 is a murine myeloma cell line secreting IgG2b of unknown specificity. mAb S1 and S2 (both IgM) are directed against polymorphic determinants on human Ia-like molecules (11).

Fusion Procedure and Screening of Hybrids. In fusion P₁, a BALB/c female mouse received 1.5×10^7 fresh tumor cells from patients ZV and GÖ intraperitoneally together with *Bordetella pertussis* adjuvant. 3 d later, one-third of the spleen was fused with 5×10^7 P3 \times 63-Ag8.653 myeloma cells using standard procedures (12). The cells were divided among 864 200- μl culture wells containing HAT (hypoxanthine, aminopterin, thymidine) medium and mouse peritoneal macrophages. Supernatants of hybrids were screened by the β -galactosidase cytoimmunoassay described previously (13). Hybridomas were cloned by limiting dilution.

257 hybrids were initially screened for binding on single-cell suspensions of the immunizing tumors and PMC (normal and PHA stimulated) from the tumor patients and normal individuals. The seven antibodies that reacted in this context only with the melanoma cells were cloned. On the basis of the pattern observed in tissue sections of melanomas containing normal skin, 1 antibody (mAb P3.58, IgM) of the 95 that bound to the immunizing tumors was chosen for further studies.

Immunohistochemistry. 8- μm -thick sections were dried overnight at 40°C and processed without fixation. Sections that were not used the same day were stored at -70°C . After blocking for 1 h with PBS-FCS and 100 $\mu\text{g}/\text{ml}$ normal rabbit Ig, the sections were incubated for 1.5 h in mAb, followed by three washings in PBS, each for 5 min. Peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Copenhagen) was diluted 1:50 in PBS-FCS and added for 1 h. (To remove activity against human serum proteins, 50 $\mu\text{l}/\text{ml}$ of pooled human serum was added to each antiserum stock, at least 1 d before the first use.) The sections were washed three times, fixed for 5 min with 0.07% glutardialdehyde in PBS, washed again, and stained for 20–30 min with a solution of 0.07 mg 3-amino-9-ethylcarbazol/ml PBS and 0.003% H_2O_2 . The carbazol was initially dissolved in DMSO at a 1:30 dilution. Counterstaining was performed in Mayer's hemalum for ≤ 20 s. Finally the sections were embedded in Kaiser's glycerol gelatin (E. Merck AG, Darmstadt, Federal Republic of Germany [FRG]).

Localization of staining was improved using lyophilized tissue sections treated with acetone and chloroform, each for 10 min. Sensitivity was considerably increased by incubating mAb-treated sections first with a rabbit antiserum against mouse Ig, then with a swine antiserum against rabbit Ig (both peroxidase conjugates; Dakopatts). Both antisera were diluted 1:30 in PBS, pH 7.8, containing 20% human serum, and incubated for 30 min (14). Tissue sections were blocked only in PBS-FCS.

mAb BBM.1, S1, and S2, and the tissue culture supernatant of the mouse myeloma line, MPC11, served as controls. All antibodies were used as undiluted tissue culture supernatants dialyzed against PBS.

Immunoprecipitation. Mel Ei melanoma cells were surface iodinated using the glucose oxidase-lactoperoxidase method as described (15). Cells were lysed ($5 \times 10^7/\text{ml}$) in PBS containing 18 mM taurocholic acid, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM

benzamidinium hydrochloride, and 200 U/ml trasylol (Bayer AG, Leverkusen, FRG), pH 8.0 (lysing buffer) for 30 min at 4°C. The nuclei were removed by centrifugation and the lysate was precleared by incubation with horse serum conjugated to Sepharose CL-4B. To prepare immunosorbents, 40 µl protein A-Sepharose CL-4B were incubated with 0.5 ml of a 1:10 dilution of rabbit anti-mouse IgM (Nordic Lab., Tilburg, The Netherlands) in PBS pH 8.0, washed, and incubated with 15 ml of tissue culture supernatant containing mAb. Precleared lysates were incubated with the immunosorbents, with rotation, for 12 h at 4°C. The bound immune complexes were eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (with or without 2-mercaptoethanol) and analyzed on a 10% SDS-PAGE (16). Fixed, stained, and dried gels were exposed to Kodak XAR-5 film at -70°C with an intensifying screen. Molecular weight markers (CFA.626) were obtained from Amersham International, Buckinghamshire, England.

Western Blotting. The gp75 antigen was isolated from a lymph node metastasis of malignant melanoma (DAF) by incubation of a precleared lysate prepared from 1 g tissue (homogenized in lysing buffer) with mAb 15.75 bound to Sepharose-protein A. The antigen was eluted by boiling in SDS sample buffer and separated on 10% SDS-PAGE under reducing conditions. Separated proteins were electrophoretically transferred to nitrocellulose membranes (BA 85; Schleicher & Schüll, Dassel, FRG) as described (17, 18). The filters were incubated with mAb 15.75 or control supernatant (myeloma MPC11) followed by peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts). Bound antibody was visualized with the addition of a substrate solution of 1.26 mM *o*-dianisidine hydrochloride, 15.3 mM sodium nitroferricyanide, and 0.007% H₂O₂.

Results

Characterization of Antigens Recognized by mAb 15.75 and P3.58 and Analysis of Their Expression on Fresh Tissues. As previously reported (5), mAb 15.75 defines a 75,000 (75 K) mol wt glycoprotein expressed on the surface of human melanoma cell lines. As shown by Western blot analysis (Fig. 1), a molecule with the same molecular weight was immunoprecipitated from homogenates of a fresh metastatic melanoma. 12 of 15 melanomas examined in tissue sections expressed

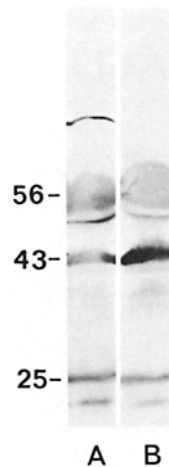


FIGURE 1. Western blot of gp75 antigen isolated from melanoma metastasis DAF. Immunoprecipitation, SDS-PAGE, and blotting are described in Materials and Methods. Nitrocellulose filters were incubated with mAb 15.75 (A) or control MPC11 myeloma (B), followed by peroxidase-coupled rabbit anti-mouse Ig. The bands observed in both lanes at 56, 43, and 25 mol wt are from the antibody 15.75 used in the immunoprecipitate.

gp75 as determined by immunoperoxidase staining with mAb 15.75, whereas no reactivity was observed with a variety of other carcinomas and benign proliferative lesions (Table I). No reactivity was observed with normal structures of the skin (epidermis, hair follicles, sweat glands, sebaceous glands), intestine, liver, and nerves. Blood cells (red cells, granulocytes, normal and PHA-stimulated mononuclear cells) and bone marrow cells from seven normal transplant donors also showed no reactivity (Table I).

From a surface-iodinated melanoma cell line, mAb P3.58 immunoprecipitated proteins with an approximate molecular weight of 89 K under nonreducing conditions, and 93 K under reducing conditions (Fig. 2). In histological sections, mAb P3.58 reacted with 13 of 15 melanomas and 3 of 16 carcinomas tested. In contrast to the melanomas, all three positive carcinomas of the gastrointestinal tract contained only 5–10% positive tumor cells, much less than seen in any of the melanomas. No reactivity was observed with several other tumors and benign proliferative lesions, nor with normal structures of the skin, intestine, liver, or nerves (Table I). Occasional positive reactions with blood vessel endothelia were observed, but no correlation could be made to a certain type of blood vessel. Blood cells and bone marrow cells from seven normal transplant donors were negative (Table I).

Comparison of Antigen Expression on Melanomas and Nevi. 15 malignant melanomas, both primary tumors (superficial spreading and nodular melanomas) and metastases, and 21 nevi (19 normal hyperplastic nevi and 2 nevi with cytologic atypia) were investigated for the expression of five different surface antigens defined by mAb.

12 out of 15 melanomas tested were positive with both mAb 15.75 and P3.58.

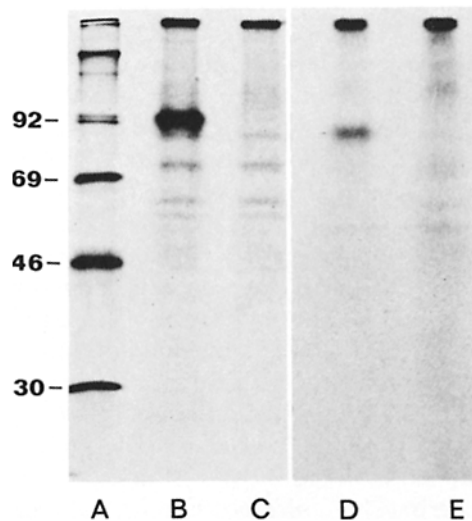


FIGURE 2. Autoradiogramm of immunoprecipitates from the surface-iodinated melanoma cell line Mel Ei. Samples were run on a 10% SDS-PAGE. (A) molecular weight markers, (B) antigen precipitated by mAb P3.58, reduced, (C) molecules precipitated by rabbit anti-mouse IgM, reduced, (D) antigen precipitated by mAb P3.58, nonreduced, (E) molecules precipitated by rabbit anti-mouse IgM, nonreduced.

TABLE I
Reactivity of mAb With Tumors and Normal Tissues

Tissue	gp75*	p89†
Melanomas	12/15‡	13/15
Carcinomas		
Breast	0/7	0/7
Stomach	0/1	1/1
Intestine	0/2	2/2
Liver	0/1	0/1
Adrenal cortex	0/1	0/1
Thyroid gland	0/1	0/1
Basal cell	0/3	0/3
Neuroblastoma	0/1	0/1
Fibrosarcoma	0/1	0/1
Nevi	1/21	2/19
Goiter	0/2	0/2
Neurofibroma	0/1	0/1
Fibroadenoma (breast)	0/1	0/1
Bone marrow	0/7	0/7
Blood cells	0/5	0/5
Liver	0/2	0/2
Intestine	0/3	0/3

Reactivity was determined by evaluation of immunoperoxidase staining of tissue sections as described in Materials and Methods.

* Defined by mAb 15.75.

† Defined by mAb P3.58.

‡ Number of positive samples per number tested. Peripheral nerves and blood vessels that were observed in many tissues showed no reactivity. Occasional reactivity of mAb P3.58 with endothelial cells was observed (see text).

mAb P3.58 reacted with one additional melanoma (Table II). Both antibodies recognized only a subpopulation of tumor cells, which varied between 10 and 50% for mAb 15.75, and 20 and 80% for P3.58 (Fig. 3, *a* and *c*). Analysis of sequential sections indicated that the positive reactivity was confined to discrete three-dimensional nests of tumor cells. Reactivity of both antibodies sharply outlined the individual cells (Fig. 3, *a* and *c*). This staining pattern is consistent with the location of the antigens on the cell surface. That both the gp75 and the p89 antigen are products of the melanoma cells themselves is supported by studies on cloned melanoma cell lines in vitro. Both antigens are located on the surface of these cells, as demonstrated by immunofluorescence staining and labeling of the molecules by surface iodination. In addition, both antigens are labeled by incorporation of radioactive amino acids (Fig. 2; reference 5 and unpublished data). Melanoma cells invading epidermis or hair follicles were consistently negative for both antigens. No distinction, by reactivity with either antibody, could be made between different histological types of melanomas nor between primary or metastatic tumors. In contrast, the majority of nevi examined were negative with both antibodies (Fig. 3, *b* and *d*). Brown granules on some nevus cells represent typical melanin deposits. Of 21 normal hyperplastic and atypical nevi, only a single junctional nevus showed reactivity with both antibod-

TABLE II
Reactivity of mAb With Melanomas

Melanomas	gp75*	p89*	Ia-like [‡]	p97 [§]
Primary tumors				
SSM [¶]				
2316, LI [¶]	-	-	+	+
2199, LII	+	+	+	+
2166, LIII	+	+	+	+
2183, LIII	+	+	+	NT**
2202, LIII	+	+	+	+
2215, LIII	-	-	+	-
2234, LIII	-	+	+	+
GRU, LIV	+	+	+	+
NM ^{**}				
2242, LIV	+	+	+	+
2164, LIV	+	+	+	NT
2189, LIV	+	+	+	NT
HUK, LV	+	+	+	NT
Metastases				
Lymph node				
DAF	+	+	+	NT
PET	+	+	+	+
Skin, 2273	+	+	+	+

* See Table I.

[‡] Defined by mAb 691-13-17.

[§] Defined by mAb 96.5.

[¶] Superficial spreading melanoma.

[¶] Level of tumor invasion according to Clark (41).

** Not tested.

** Nodular melanoma.

ies. mAb P3.58 reacted with one additional intradermal nevus (Table III). Using the more sensitive immunohistochemical technique described in Materials and Methods, we did not observe any difference in the staining pattern of mAb 15.75 and P3.58 on melanomas, nor reactivity with nevi.

mAb 96.5, directed against the transferrin-related molecule p97, reacted with 9 of 10 melanomas tested and 16 of 17 nevi (Tables II and III), which is consistent with previous reports (19). In addition, we observed staining of sweat glands and the basal cell layer of hair follicles.

Both melanomas (three of three) and nevi (seven of eight) showed expression of the transferrin receptor as defined by mAb 5E9 (Table III). Strong reactivity was also observed with epidermis, hair follicles, sweat glands, and sebaceous glands. Smooth muscle, blood vessels, and connective tissue were found to be negative.

Using two mAb against monomorphic determinants on human Ia-like antigens, all melanomas examined expressed these molecules (Table II). Expression of Ia-like antigens was also observed on nevus cells in all nevi (Table III). The Langerhans cells in the epidermis showed the characteristic strong expression of Ia-like antigens (20) and similar cells were observed in the dermis between the nests of nevus cells.

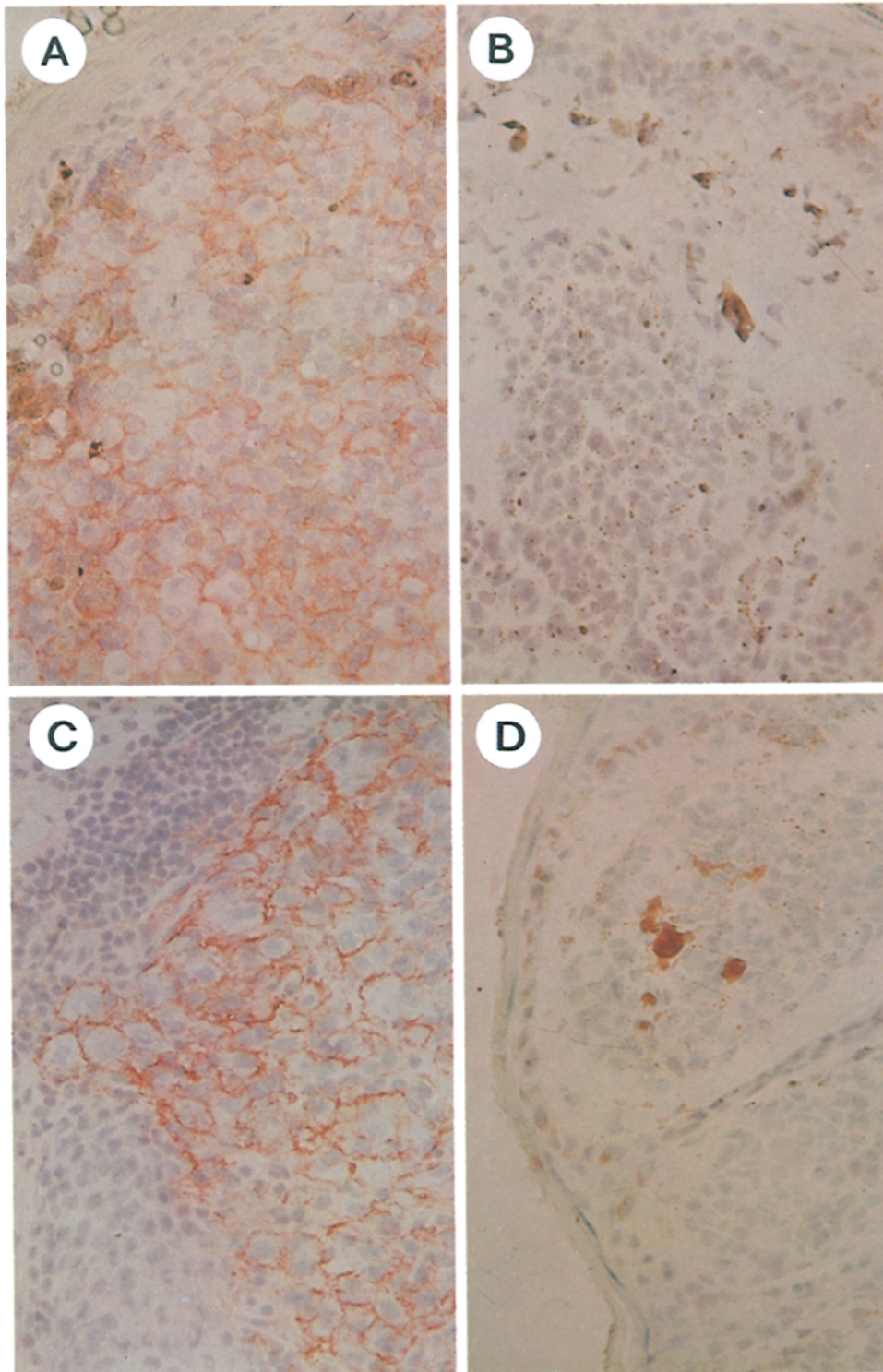


FIGURE 3. Immunoperoxidase staining of melanomas and nevi with different mAb. $\times 160$ (mAb P3.58), $\times 160$ (mAb 15.75). (A) melanoma GRU, mAb 15.75, (B) intradermal nevus, mAb 15.75, (C) melanoma GRU, mAb P3.58, (D) intradermal nevus, mAb P3.58.

TABLE III
Reactivity of mAb With Nevi

Nevi	gp75*	p89*	p97*	TR [†]	Ia-like*	Ia-like [‡]
Common acquired nevi						
Junctional	1/4 [‡]	1/4	3/3	NT [‡]	3/3	NT
Compound	0/5	0/4	3/4	3/4	5/5	4/4
Intradermal	0/9	1/8	8/8	4/4	9/9	4/4
Spindle cell	0/1	0/1	1/1	NT	1/1	NT
Nevi with cytologic atypia						
Atyp Mel Hyp**	0/1	0/1	1/1	NT	1/1	NT
Atyp Nev Len ^{‡‡}	0/1	0/1	NT	NT	1/1	NT

* See Table I and II.

[†] Transferrin receptor defined by mAb 5E9.

[‡] Defined by mAb DA6.231.

^{‡‡} See description of data in Table I.

[‡] Not tested.

** Atypical melanocytic hyperplasia.

^{‡‡} Atypical nevoid lentigo.

Discussion

The melanocyte lineage provides a unique system for the study of changes occurring during the development of spontaneous solid tumors in man (3, 30). While normal melanocytes are scattered through the basal cell layer of the epidermis, the benign proliferation of melanocytes and their organization in clusters gives rise to a nevus. The majority of melanomas are thought to develop through the malignant transformation of melanocytes in preexisting nevi (3, 21). Clark et al. (3) recently described the evolution of malignant melanoma as a sequence of several alterations of melanocyte growth, beginning with the focal proliferation of benign cells (common acquired nevus) and leading finally to the metastatic melanoma. Thus, the hypothesis of multistep tumorigenesis, originally developed in experimental tumor systems (1, 2), is supported by *in vivo* investigations on a spontaneous human solid tumor.

Numerous mAb have been produced against human malignant melanoma (5, 22–27). While several of these melanoma-associated antigens are not detectable on melanocytes (19, 28–30), all markers examined are expressed on benign nevi as well as melanomas (19, 26, 28, 29, 31). The focus of this study was to define, on a molecular basis, differences between the clearly benign (but activated) and the clearly malignant phenotypes of melanocytes. To this end, we looked for mAb that could distinguish between common acquired nevi and malignant melanoma.

Malignant melanomas have been reported to express Ia-like antigens (32, 33), transferrin receptor (34), and the transferrin-related molecule, p97 (19). While Ia-like antigens were not detected on skin melanocytes either *in situ* (20) or after short-term culture (30) (adult and fetal), they have been reported on nevus cells both in short-term culture (31) and *in situ* (28). In the present study, using two different mAb directed against monomorphic determinants on human Ia-like antigens, all nevi were found to contain positive nevus cells and a substantial fraction of cells expressing the transferrin receptor. Since the expression of the transferrin receptor has been shown (9, 34) to be associated with cell proliferation

and activation, these observations confirm the view that nevi contain populations of activated and proliferating pigment cells. As nevi are essentially benign lesions, neither the expression of transferrin receptor nor of Ia antigens mark the transition to the transformed state.

In contrast, the two antigens, gp75 and p89, as defined by mAb 15.75 and P3.58, respectively, appear to be differentially expressed on malignant melanomas and their nonmalignant precursor lesions. Whereas 12 of 15 melanomas showed reactivity with both antibodies, only 1 of 21 nevi was reactive. Analysis of serial sections confirmed that each antibody reacted only with a subpopulation of tumor cells, as reported with mAb directed to other antigens (29, 35). It appears that the tumor cell populations recognized by each antibody are usually not overlapping. The absence of these antigens on the activated nevus cells suggests that they are correlated with the appearance of the malignant phenotype of melanocytes, although the correlation is not absolute, since one nevus reacted with both mAb 15.75 and P3.58, and one additional nevus only with mAb P3.58. Whether the molecules defined by these two mAb are required for malignant transformation cannot be answered by antibody binding, and will require further biochemical and molecular biological studies. However, it is possible that changes usually connected with malignant transformation appear on morphologically nonmalignant tissue, if other events necessary for transformation are missing. Since this study focused on histologically normal nevi, the antigen expression of intermediate phenotypes like dysplastic nevi requires further investigation.

Recent observations on oncogenes indicate that malignant cells may indeed differ from their normal counterpart, not only by quantitative or temporal variation in the expression of certain molecules (36, 37), but in the expression of structurally altered molecules (38, 39). Thus, while many quantitative and qualitative differences are detected on the mRNA level using a cDNA subtraction technique (40), discrete structural alterations may best be defined by mAb. The selection of mAb for differential reactivity on malignant vs. benign hyperplastic lesions of the same lineage may be a useful approach in detecting such molecules.

Summary

Monoclonal antibodies (mAb) were selected for differential binding to sections of freshly frozen biopsy material of human malignant melanomas and their precursor lesions, the melanocytic nevi. Both melanomas and normal nevi expressed human Ia-like antigens, transferrin receptor and the transferrin-related molecule p97. In contrast, only 1 nevus of 21 tested expressed both glycoprotein gp75, defined by mAb 15.75, and protein p89, defined by mAb P3.58, whereas 12 of 15 melanomas tested expressed both antigens. mAb P3.58 reacted with one additional melanoma and one nevus. The expression of these two molecules therefore appears to be correlated with the appearance of the malignant phenotype of melanocytes.

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