SURFACE ANTIGENS OF MELANOCYTES AND MELANOMAS

Markers of Melanocyte Differentiation and Melanoma Subsets*

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Knowledge about the surface antigens of malignant melanoma has grown rapidly since the advent of monoclonal antibodies (1–5). A large number of cell lines derived from melanomas have now been established, and these have facilitated the serological analysis of melanoma surface antigens. Many of the melanoma antigens that have been identified with mouse monoclonal antibodies are not expressed by all melanoma lines but show instead a differential pattern of expression that defines melanoma subsets on the basis of surface antigenic phenotype. It seems likely that this diversity of melanoma phenotype reflects a corresponding diversity in the surface phenotype of normal cells undergoing melanocyte differentiation. To pursue this idea, we analyzed the surface antigens of melanocytes, using a recently described method for growing melanocytes from normal skin (6). Most, but not all, of the antigens initially detected on melanomas were also detected on melanocytes, and the pattern of antigen expression on newborn and adult melanocytes could be distinguished. From these studies of melanocytes and melanomas, we propose a scheme of surface antigenic changes occurring during melanocyte differentiation and a classification of melanoma based on expression of melanocyte differentiation antigens.

Materials and Methods

Melanocyte Cultures. Skin from face, trunk, or thigh of 16 adults, foreskin of 14 newborns, and skin from the trunk of a 12-wk fetus served as the source of melanocytes. Cultures of melanocytes were grown in the presence of the phorbol ester, TPA (12-O-tetradecanoyl-phorbol-13-acetate) 10 ng/ml (Consolidated Midland Corp., Brewster, NY), and 1×10^{-8} M cholera toxin (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY), according to previously published procedures (6).

Melanoma Cultures. For derivation of melanoma cell lines, see ref 7. Cultures were maintained in Eagle's minimum essential medium supplemented with either 10% fetal bovine serum (FBS) or 8% newborn calf serum mixed with 2% FBS, 2 mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were regularly tested for mycoplasma, and contaminated cultures were discarded. Five melanoma cell lines were tested between passage one and three (SK-MEL-65,163,164,165,173), while the remaining cell lines were tested after passage 8, usually between passages 15 and 50. In addition, eight melanoma cell lines (SK-MEL-13,19,28,37,64,127,130, and MeWo) were grown in the presence of TPA (10 ng/ml) and cholera toxin (1×10^{-8} M) for 2–3 wk to determine the effect of these growth factors on surface antigen expression.

Serological Procedures. The anti-mouse immunoglobulin (anti-Ig) assay and protein A (PA)

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assays were performed as described previously (3, 8). Indicator cells were prepared by conjugating purified anti-mouse immunoglobulin (Dako Corp., Santa Barbara, CA) or protein A (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) to human erythrocytes using 0.01% chromium chloride. Assays were performed in Falcon 3040 Microtest II plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). Sera were incubated with target cells for 1 h at 20°C. Target cells were then washed, and indicator cells were added for 1 h. Plates were washed and evaluated for rosetting by light microscopy. Titers corresponded to the serum dilution giving 50% positive target cells. Absorption tests were carried out as described (3, 7, 8).

Serological Reagents (See Table I). Monoclonal antibodies were derived from spleen cells of mice immunized with melanoma, glioma, renal cancer, or melanocyte cell cultures (3, 9, 10; A. Houghton and A. Albino, unpublished results). *nu/nu* mice (Swiss background) were inoculated with cloned hybridomas, and sera from tumor-bearing mice were used for serological analysis.

TABLE I

Panel of Mouse Monoclonal Antibodies Detecting Cell Surface Antigens of Melanomas and Melanocytes

Cell surface antigen system	Original designation of monoclonal antibody	Immunizing cell type	Reference
M-1	L1-27	Melanoma SK-MEL-33	
M-2	A010	Glioma SK-AO-2	10
M-3	A092	Glioma SK-AO-2	10
M-4	M111	Cutaneous melanocytes	
M-5	M231	Cutaneous melanocytes	
M-6	AJ8	Glioma AJ	10
M-7	AJ17	Glioma AJ	10
M-8	D14	Melanoma DX-2	
M-9	Mel-1*		12
M-10	M144	Cutaneous melanocytes	
M-11	A1-27	Melanoma SK-MEL-19	
M-12	L166	Melanoma SK-MEL-33	
M-13	E20	Melanoma SK-MEL-93	
M-14	AJ225	Glioma AJ	10
M-15	AJ9	Glioma AJ	10
M-16	K1114	Melanoma SK-MEL-31	
M-17	AJ60	Glioma AJ	10
M-18	R_{24}	Melanoma SK-MEL-28	3
M-19	I_{12}, L_{10}, K_5, L235	Melanoma SK-Mel-28,33	3
M-20	L101, Q_{24}, Q_{14}, 829, 846	Melanoma SK-MEL-28,33,93	3
M-21	I_{24}	Melanoma SK-MEL-28	3
M-22	R_8	Melanoma SK-MEL-28	3
M-23	L2-30, L254	Melanoma SK-MEL-33	
M-24	M138	Cutaneous melanocytes	
M-25	M3-68	Cutaneous melanocytes	
M-26	A123	Melanoma SK-MEL-19	
M-27	A124	Melanoma SK-MEL-19	
M-28	B5	Melanoma SK-MEL-93	
M-29	AJ2	Glioma AJ	10
M-30	AJ10	Glioma AJ	10
M-31	A050	Glioma SK-AO-2	10
M-32	A0122	Glioma SK-AO-2	10
M-33	V_2	Renal cancer SK-RC-6	9
M-34	S_6	Renal cancer SK-RC-7	9

* Defined by naturally occurring antibody in human serum that detects the Mel-1 melanocyte differentation antigen (see Materials and Methods).

These sera had maximum titers of $\geq 10^{-4}$, and usually $>10^{-5}$, with melanoma or melanocyte cultures. With the exception of M-18 antibody, which was detected by PA assays, all other mouse monoclonal antibodies were analyzed using the anti-Ig assay. Monoclonal antibodies known to identify the same epitope or different epitopes on the same molecule were grouped under the same antigenic system; M-19 antigen is a 95-kd glycoprotein detected by four mouse monoclonal antibodies, M-20 represents a 130-kd glycoprotein detected by five different mouse monoclonal antibodies, and M-23 is a glycoprotein dimer of 145 kd and 100 kd detected by two antibodies. Mouse monoclonal antibody and conventional rabbit antisera to β2-microglobulin (018b) were purchased from Accurate Chemical and Scientific Corp., Westbury, NY. Serological and biochemical characteristics of the human HLA-DR monoclonal antibody 13–17 have been published (1, 11). Antigen M-9 was detected by a naturally occurring antibody in the serum of a healthy individual; this antibody defines a melanocyte differentiation antigen, Mel-1, which is expressed by ~50% of melanoma cell lines (12).

Tyrosinase Assay. Tyrosinase activity was measured using a modification of the assay described by Pomerantz (13). [^{3}H]Tyrosine (specific activity, 53.1 Ci/mMol) was purchased from New England Nuclear (Boston, MA). Cell lines to be tested were seeded at a density of 1 $\times$ 10^6 cells per flask in 25 cm^2 Falcon flasks. After 12 h, medium was removed and replaced with 4 ml fresh medium containing 5 μCi [^{3}H]tyrosine, and the cultures were incubated for an additional 24 h. Residual [^{3}H]tyrosine in the medium was removed by adsorption to activated charcoal and passage over a Dowex 50w column (Bio-Rad Laboratories, Richmond, CA). Tritiated H_2O (generated by tyrosinase activity) in the eluent was counted in triplicate in a LS 9000 Beckman Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA). Tyrosinase activity was expressed as the ratio of tritiated H_2O generated by melanoma cell lines per tritiated H_2O generated by a control nonpigmented renal cancer cell line (SK-RC-7).

Results

Description of Antibodies Reactive with Cell Surface Antigens of Melanoma and Melanocytes. The panel of typing antibodies used in this study is described in Table I, and reactivity and titer of these antibodies with melanoma cell lines are shown in Table II. Fig. 1 illustrates individual assays with antibodies detecting M-4, M-10, and HLA-DR antigens tested on three melanoma cell lines and two melanocyte cultures.

A characteristic of the typing antibodies listed in Table I is that they generally react with only a proportion of the melanoma cell lines and therefore divide melanomas into distinguishable subsets on the basis of antigenic phenotypes. Antigens such as M-25 are expressed by most melanoma lines (23/26 lines), antigens M-4 and M-9 are detected on approximately one-half of the cell lines, and M-10 antigen is found on only 5 of 33 melanoma lines. With regard to major histocompatibility complex (MHC) products, HLA-DR expression was found on 13 of 21 cell lines. Antibodies to HLA-A,B,C and β2-microglobulin were highly reactive with nearly all melanoma cell lines; two lines, SK-MEL-19 and SK-MEL-33, showed no reaction. By absorption tests, these antigens were detectable on SK-MEL-19 but not on SK-MEL-33.

Serological Typing of Fetal, Newborn, and Adult Melanocytes. Fig. 2 shows the morphology of melanocytes from newborn and adult skin. Fetal and newborn melanocytes grow as bipolar cells. In contrast, melanocytes from adult skin show a polydendritic morphology. Fig. 3 summarizes the results of serological tests with newborn and adult melanocytes. The reactivity of melanocytes derived from fetal skin was similar to newborn melanocytes.

Melanocyte antigens detected by the panel of typing antibodies can be grouped into four categories: (*a*) not detected on newborn or adult melanocytes but expressed by subsets of melanoma: HLA-DR, M-1, M-2, and M-3; (*b*) detected on newborn melanocytes but not adult melanocytes: M-4 to M-8; (*c*) detected on adult melanocytes

TABLE II

Serological Typing of Melanoma Cell Lines by Mouse Monoclonal Antibodies

Cell surface antigen system	Melanoma cell lines reactive/tested (titer range)
HLA-A,B,C	9/11 (10^{-5}–10^{-6})
β_2-microglobulin	37/38*
HLA-DR	13/21 (10^{-3}–10^{-5})
M-1	5/32 (10^{-4}–10^{-5})
M-2	6/28 (10^{-3}–10^{-5})
M-3	17/31 (10^{-3}–10^{-4})
M-4	16/33 (10^{-4}–10^{-7})
M-5	10/23 (10^{-3}–10^{-5})
M-6	12/31 (10^{-3}–10^{-5})
M-7	10/10 (10^{-4}–10^{-5})
M-8	17/18 (10^{-4}–10^{-5})
M-9	22/43 (10^{-2}–10^{-5})
M-10	5/33 (10^{-3}–10^{-4})
M-11	19/33 (10^{-4}–10^{-6})
M-12	16/19 (10^{-4}–10^{-6})
M-13	15/15 (10^{-5}–10^{-6})
M-14	3/13 (10^{-3}–10^{-5})
M-15	10/10 (10^{-4}–10^{-5})
M-16	10/34 (10^{-3}–10^{-5})
M-17	16/16 (10^{-3}–10^{-5})
M-18	16/16 (10^{-3}–10^{-5})
M-19	20/29 (10^{-4}–10^{-6})
M-20	24/27 (10^{-5}–10^{-6})
M-21	16/16 (10^{-3}–10^{-5})
M-22	9/16 (10^{-4}–10^{-6})
M-23	19/19 (10^{-5}–10^{-7})
M-24	22/26 (10^{-5}–10^{-7})
M-25	23/26 (10^{-5}–10^{-6})
M-26	17/19 (10^{-4}–10^{-7})
M-27	14/33 (10^{-3}–10^{-6})
M-28	9/10 (10^{-4}–10^{-5})
M-29	10/10 (10^{-5}–10^{-6})
M-30	9/10 (10^{-5}–10^{-6})
M-31	8/10 (10^{-4}–10^{-6})
M-32	8/10 (10^{-4}–10^{-6})
M-33	6/6 (10^{-5}–10^{-6})
M-34	2/30 (10^{-3}–10^{-4})

* Determined by absorption tests; rabbit antihuman β_2-microglobulin (diluted according to endpoint) was absorbed with individual melanoma cell lines and residual antibody activity tested against a standard melanoma target cell line (SK-MEL-28).

and only weakly or not at all on most newborn melanocytes: M-9 and M-10; and (*d*) detected equally on both newborn and adult melanocytes: M-11 to M-34. Most of the antigens in this last group were also expressed by the majority of melanoma cell lines. M-34 antigen was an exception in this regard, as it was detected on only 2/30 melanoma cell lines, as compared with 14/14 fetal, newborn, and adult melanocyte cultures. The monoclonal antibody recognizing M-34 detects a 120,000 M_r glycoprotein that is expressed by renal cancer, normal kidney epithelium, and a limited number of nonrenal cells (9).

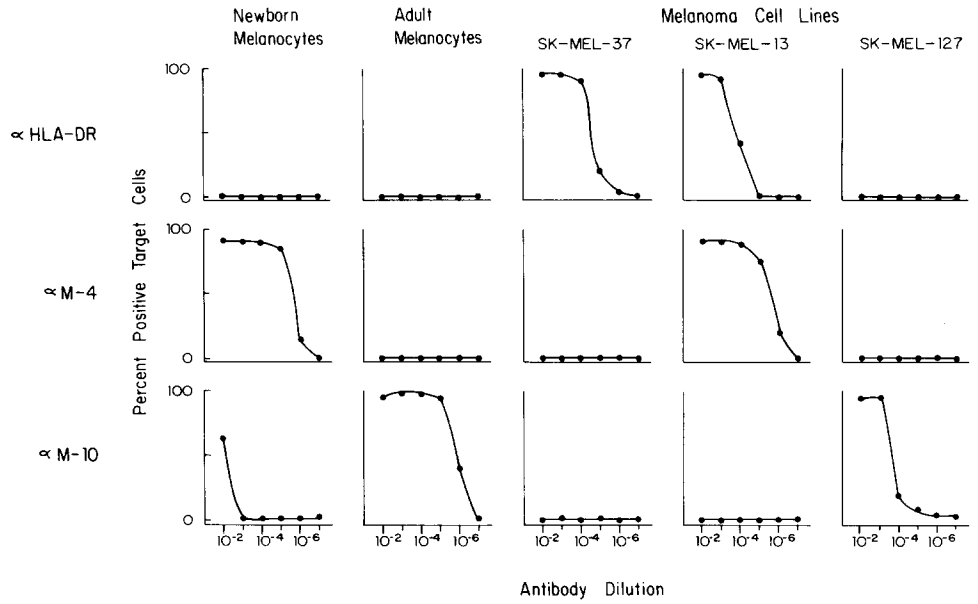

FIG. 1. Serological typing of melanocytes and melanoma cells for HLA-DR, M-4, and M-10 cell surface antigens. Anti-Ig assay.

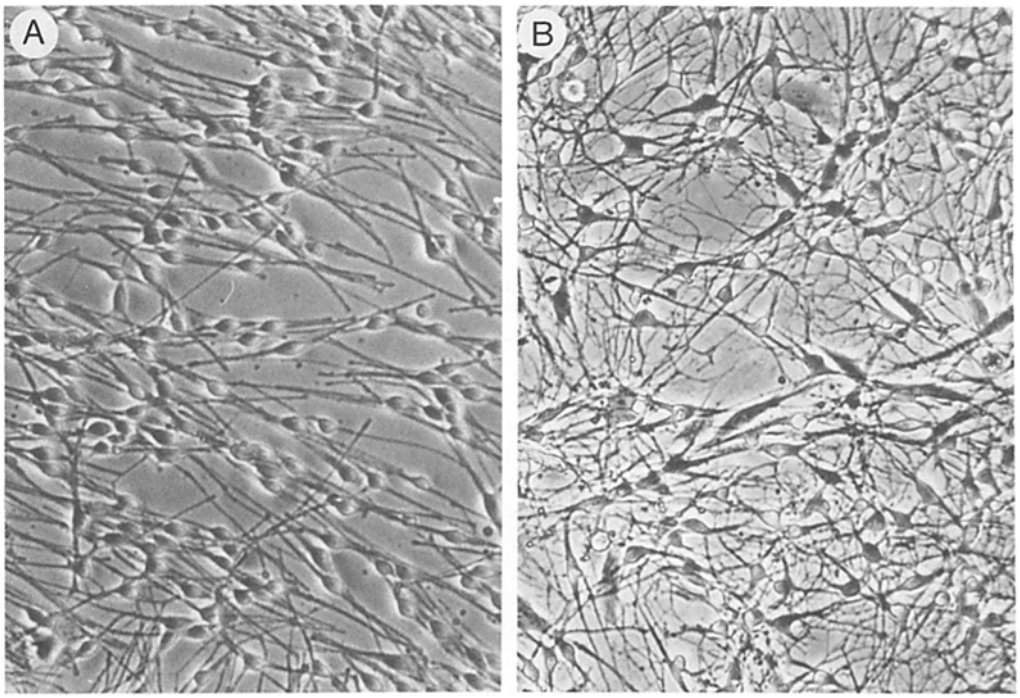

FIG. 2. Morphology of melanocytes from newborn foreskin (*A*) and adult skin (*B*). Newborn melanocytes have a bipolar morphology, whereas adult melanocytes are polydendritic. Magnification, 360 ×.

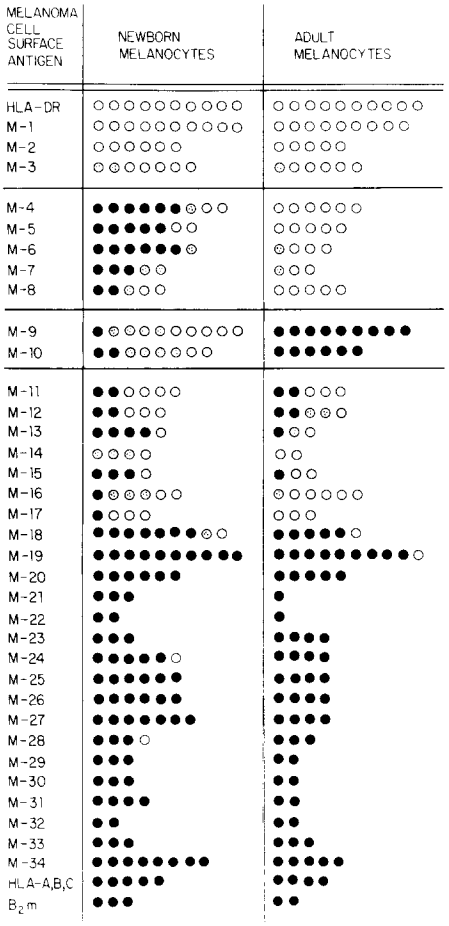

FIG. 3. Serological typing of newborn and adult melanocytes for melanoma cell surface antigens. Each circle represents an individual test, and each test for a particular antigen was performed with melanocytes from a different individual. In the case of tests with mouse monoclonal antibodies, black circles represent antibody titers $1:10^4$ to $1:10^7$, stippled circles 1:500 to 1:5,000, and open circles $\leq$1:250. In tests with human typing serum detecting M-9 antigen, black circles represent titers 1:500 to $1:10^4$, stippled circles 1:10 to 1:250, and open circles, no reaction. (<1:10).

24 other monoclonal antibodies detecting a series of differentiation antigens on renal cancer, lung cancer, and ovarian cancer and ABH blood group antigens were included in these tests. No reactions with newborn or adult melanocytes were observed.

Influence of In Vitro Culture Conditions on Antigen Expression. Melanocytes require TPA and cholera toxin for at least the first 4 d of culture to stimulate melanocyte proliferation and to inhibit overgrowth of keratinocytes and fibroblasts. However, after this brief exposure, cultures can be maintained without growth factors for several weeks before melanocytes die or become overgrown by fibroblasts. To investigate the influence of growth factors on antigen expression, melanocytes were grown for 4 d in TPA and cholera toxin conditioned media and then for 3 wk in (*a*) culture medium without growth factors, (*b*) culture medium with either TPA or cholera toxin, or (*c*) culture medium with both TPA and cholera toxin. We did not observe any difference in antigen expression when melanocytes were cultured under these various conditions;

monoclonal antibodies detecting antigens M-1 through M-6, M-9, M-10, and M-34 were used in these tests. Studies were also carried out to determine whether melanoma cell lines grown in the presence of TPA and cholera toxin would change their surface antigenic phenotype. Of the 28 antigenic systems tested on 8 melanoma cell lines, only M-34 antigen was influenced by the presence of growth factors; SK-MEL-19, which characteristically expresses little or no M-34 antigen, converted to M-34$^+$ after growth in TPA and cholera toxin.

To reduce the possibility that length of time in culture might alter antigen expression, most tests were carried out with melanocyte cultures between passage 1 and 3. However, little difference in antigen expression was seen when melanocytes were tested later at passage 8, 12, or 19. M-4 and M-5 antigens, which are markers of newborn and fetal melanocytes, appear to be exceptions because they could not be detected on newborn melanocytes after five passages in culture.

Subsets of Melanoma Cell Lines Defined by Melanocyte Differentiation Antigens. 25 melanoma cell lines were typed for the expression of M-2, M-3, HLA-DR, M-4, M-6, M-9 and M-10 antigens. These seven antigens were selected because they defined subsets of melanomas (present on some melanomas but not others) and had distinct patterns of expression on fetal/newborn and adult melanocytes. Three antigens, HLA-DR, M-2, and M-3, can be assumed to be early markers of melanocyte differentiation because they are expressed on melanomas but not on melanocytes. Antigens M-4 and M-6 appear on fetal and newborn melanocytes but not adult melanocytes and, therefore, signal an intermediate phase in melanocyte differentiation. M-9 and M-10 appear to be late markers in the melanocyte lineage because they are strongly

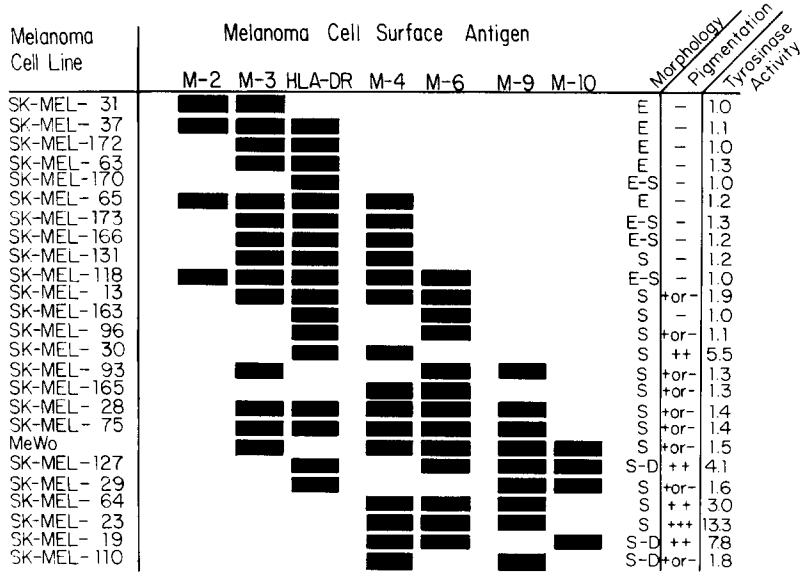

FIG. 4. Serological typing of 25 melanoma cell lines for melanocyte differentiation markers. Black rectangles represent antigen expression by melanoma cell lines, as determined by titers of 1:500 to $1:10^7$ for mouse monoclonal antibodies and 1:10 to $1:10^5$ for human serum detecting M-9 antigen. Morphology: E, epithelioid; S, spindle-shaped; D, polydendritic. Pigmentation was estimated visually by the intensity of brown or black pigment in the cell pellet. Tyrosinase activity was expressed as a ratio of tritiated H_2O produced by melanoma culture/tritiated H_2O produced by nonpigmented renal cancer culture (standard).

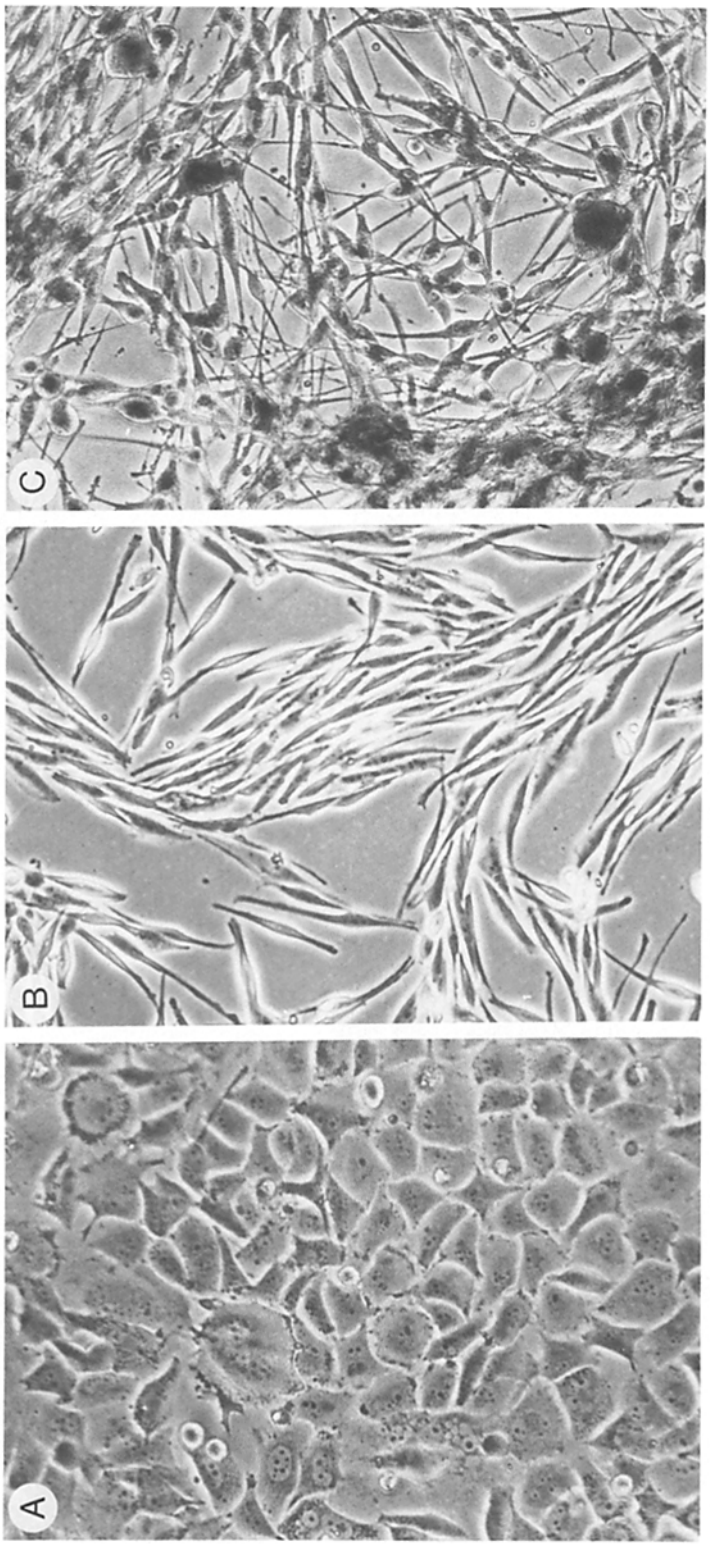

FIG. 5. Morphology of melanoma cell lines expressing early, intermediate, or late melanocyte surface markers. Magnification, 220 ×. (A) SK-MEL-37 is representative of lines with early melanocyte markers, and melanoma cells in these cultures have an epithelioid morphology and no pigmentation. (B) MeWo is representative of lines with intermediate melanocyte markers, and melanoma cells in these cultures have a bipolar, spindle-shaped morphology with little or no pigmentation. (C) SK-MEL-23 represents lines with late melanocyte markers. Heavily pigmented cells with long, dendritic processes are characteristic of melanoma cells in these cultures.

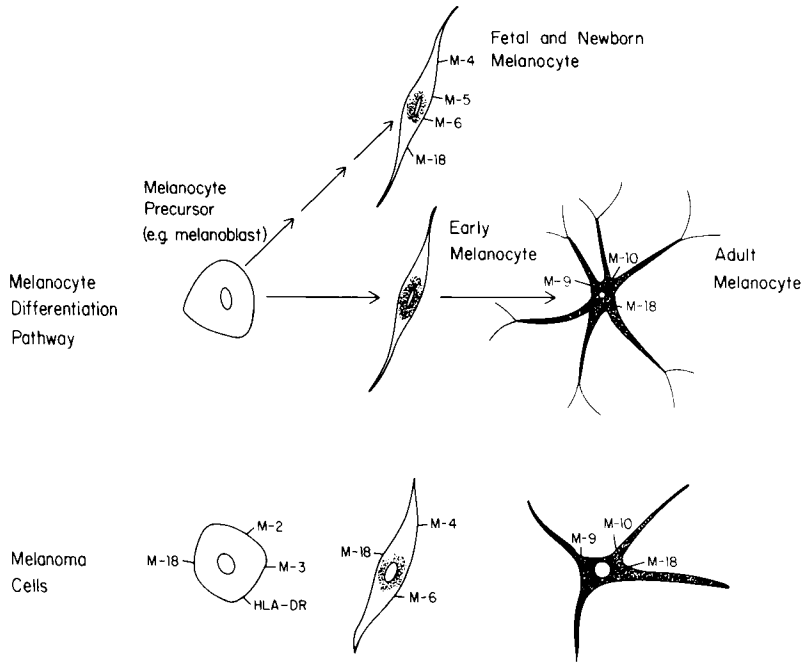

FIG. 6. Proposed pathway of melanocyte differentiation based on surface antigenic phenotype and morphology. The phenotype of melanomas corresponding to early, intermediate, or late stages in the proposed melanocyte pathway is also illustrated. In this scheme, M-2, M-3, and HLA-DR antigens are early melanocyte markers, M-4, M-5, and M-6 antigens are intermediate markers, and M-9, and M-10 antigens are late melanocyte markers. M-18 antigen is found at all stages of melanocyte differentiation and is present on all melanoma cells.

expressed on adult melanocytes as compared with fetal and newborn melanocytes.

The surface phenotypes of the 25 melanoma lines appear to correspond to early, intermediate, or late phases of melanocyte differentiations. 5 melanomas expressed only early markers, 10 intermediate markers, and 10 late melanocyte markers (Fig. 4). Evidence for the significance of these differences comes from a comparison of the pattern of surface antigens with other differentiation characteristics, such as pigmentation, morphology, and tyrosinase (Fig. 4). The majority of melanomas expressing early antigenic markers are epithelioid (Fig. 5 a), and these melanoma lines lack pigmentation and tyrosinase activity. Fig. 5 b shows the morphology of a cell line belonging to the intermediate group, and the resemblance to the bipolar morphology of melanocytes from fetal and newborn skin is apparent. Melanomas expressing late melanocyte markers frequently have a polydendritic morphology similar to adult melanocytes, with heavy pigmentation and high levels of tyrosinase activity (Fig. 5 c).

Melanocyte Differentiation Pathway. From these studies of differentiation antigens on melanocytes and melanomas, a surface antigenic map of the melanocyte lineage can be proposed (Fig. 6). At least three distinct stages in melanocyte differentiation can be defined - precursor, intermediate, and mature. We infer the features of melanocyte precursors from the characteristics of melanomas expressing early markers of differentiation. The intermediate and mature phases of melanocyte development are defined by markers that distinguish fetal/newborn melanocytes from adult melanocytes. On the basis of surface antigens, morphology, pigmentation, and tyrosinase

activity, three classes of melanomas can be identified, corresponding to the features of normal melanocytes at the early, intermediate, or mature phases in melanocyte differentiation.

Discussion

The process of cellular differentiation is accompanied by changes in the surface antigenic phenotype, and surface antigens that distinguish cells belonging to distinct differentiation lineages or distinguish cells at different phases in the same differentiation lineage are referred to as differentiation antigens (14). Initial recognition of differentiation antigens came about through analysis of surface antigens of T cell leukemias of the mouse and the description of the TL (15), Thy-1 (16), and Lyt (17) series of antigens. The analysis of these T cell differentiation antigens was greatly simplified by the availability of normal T cells from the thymus and from other lymphoid organs for a side-by-side comparison with leukemic T cells. Although the study of differentiation antigens on T cells and B cells of mouse and man is relatively advanced, little is known about differentiation antigens displayed on normal and neoplastic cells belonging to other lineages, and this is due to the difficulty of obtaining a ready source of the appropriate normal cell type. The recently described technique to culture melanocytes from normal skin (6) provides a renewable source of proliferating cells for the analysis of melanocyte differentiation antigens, and the monoclonal antibodies detecting cell surface antigens of melanoma provide the initial serological probes for this analysis.

On the basis of reactions with melanocytes from fetal, newborn, or adult skin, we identified antigens that appear to be early, intermediate, or late markers of melanocyte differentiation. The late markers, such as M-10, are strongly expressed by adult melanocytes. Intermediate melanocyte markers, such as M-4, are found on fetal and newborn melanocytes but not adult melanocytes. With regard to antigens detected on melanomas but not on fetal, newborn, or adult melanocytes, we propose that these are early markers expressed by melanocyte precursors but not by cells further down the melanocyte pathway. Although a melanocyte precursor cell has not as yet been identified in the skin, we suggest that its surface phenotype would correspond to the M-2^+, M-3^+, HLA-DR^+ subset of melanomas. One candidate for the melanocyte precursor is the indeterminate cell type found in the basal layer of the epidermis (18). Because these cells express HLA-DR but do not contain melanosomes or tyrosinase activity, they have been considered precursors of Langerhans cells in the upper epidermis, but it is equally plausible that some indeterminate cells are precursors of melanocytes. Two other explanations for the expression of antigens on melanoma cells but not melanocytes should be considered. One possibility is that a second differentiation pathway for pigment cells exists. A bifurcation could occur in the neural crest pathway, with one arm leading to epidermal melanocytes and another arm leading to nevus cells. HLA-DR, M-2, and M-3 would be expressed only on nevus cells and pigmented tumors derived from them, but not on normal or malignant melanocytes. The other possibility, which we consider less likely, is that these antigens are unrelated to neural crest differentiation but are the result of abnormal gene expression induced during the process of malignant transformation and tumor progression.

In addition to the value that these melanocyte differentiation markers have for investigating normal melanocytes and their precursors, it seems likely that such markers will also provide new ways to analyze and classify melanomas. For instance, melanoma cell lines fall into one of three general classes on the basis of expression of

early, intermediate, or late melanocyte antigens. Although the significance of this classification awaits the results of comparable studies with noncultured tissue specimens, there is an evident correlation between the surface antigenic phenotype of the cultured melanoma line and other differentiation characteristics, such as morphology, pigmentation, and tyrosinase activity. Melanomas expressing early markers but lacking intermediate or late markers have an epithelioid morphology, lack pigmentation, and have low levels of tyrosinase. In contrast, melanomas expressing late markers, such as M-9 and M-10, have a spindle-shaped or polydendritic morphology, are pigmented, and have high levels of tyrosinase. Intermediate classes of melanoma can be distinguished that express intermediate melanocyte markers, and these generally have a spindle morphology, little pigmentation, and low levels of tyrosinase.

A question that cannot be answered with the available evidence is whether melanomas arise at any one of a number of stages throughout the melanocyte lineage or whether there is a preferential stage for malignant transformation. The finding that the phenotypes of melanoma correspond to distinct phases in the melanocyte pathway could be explained by transformation of early, intermediate, or mature progenitors, or alternatively, by transformation of early progenitors with transformants having the ability to undergo variable but characteristic degrees of differentiation to later stages of melanocyte differentiation. The overlapping phenotypic characteristics of melanoma cell lines from different individuals is consistent with either explanation. However, the striking phenotypic variation of different melanoma metastases derived from a single patient (19), where individual metastases were found to show characteristics of either early or intermediate melanocyte stages, is more consistent with a model of early stage transformation and variable capacity of progeny cells to differentiate toward later stages. This matter may be clarified by the results of current attempts to transform melanocytes from different stages of differentiation with chemical or physical carcinogens.

Summary

The surface antigens of melanocytes from newborn and adult skin have been analyzed with monoclonal antibodies detecting cell surface antigens of malignant melanoma. Antigenic markers that distinguish early, intermediate, and mature stages in melanocyte differentiation have been defined. The characteristics of the normal melanocyte precursor have been inferred from the features of melanomas that express early markers of melanocyte differentiation. A rudimentary surface antigen map of cells undergoing melanocyte differentiation and a new classification of melanomas based on the expression of melanocyte differentiation antigens are proposed.

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