CELL SURFACE ANTIGENS OF HUMAN MELANOCYTES AND MELANOMA

Expression of Adenosine Deaminase Binding Protein Is Extinguished with Melanocyte Transformation

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Clinical and histopathological observations suggest that the pathogenesis of cutaneous melanoma evolves through multiple stages. It has been proposed that early stages are characterized by benign proliferation of melanocytic cells and later stages by acquisition of competence for invasion and metastasis (1). The recognition that dysplastic nevi are a class of pigmented lesions with potential for malignant change is consistent with the existence of a precursor step in the development of melanoma (2). Phenotypic markers that discriminate normal melanocytes from melanoma cells would be particularly useful for dissecting stages in melanocyte transformation. However, definitive criteria that distinguish benign melanocytes from premalignant and malignant cells have not been established.

In previous studies, we have used morphology, pigmentation, and expression of cell surface and intracellular antigens to characterize the phenotype of cultured human melanocytes and melanomas (3, 4). Serological typing has revealed that antigens that are expressed on a subset of melanomas or melanocytes generally are regulated by the melanocyte differentiation program (3, 4). During these investigations, an antigen system was recognized whose pattern of expression was remarkable because it was not associated with the stage of melanocyte differentiation, but appeared to be regulated with melanocyte transformation. This antigen system was expressed by all cultured melanocytes but not by melanomas. mAbs that react with this antigen bind to a 120-kD cell surface glycoprotein that has been identified as the adenosine deaminase binding protein (ADAbp)[1] (5). These studies indicate that cell surface expression of ADAbp is downregulated and apparently extinguished during the process of melanocyte transformation.

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[1] *Abbreviations used in this paper:* ADAbp, adenosine deaminase binding protein; IBMX, isobutylmethylxanthine; Ha-MSV, Harvey murine sarcoma virus; Ki-MSV, Kirsten murine sarcoma virus; MuLV, murine leukemia virus.

Materials and Methods

Cell Lines and Tissue Culture. Melanoma cell lines, cutaneous and choroidal melanocyte and nevus cultures were derived by previously described techniques (6, 7). Cultures of melanocytic cells were maintained in Eagle's MEM supplemented with 2 mM glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS (complete medium). Cells were passaged with trypsin 0.25% (wt/vol) and EDTA 0.02% (wt/vol). All nevus specimens were diagnosed by standard clinical and histological criteria by the Department of Pathology, Memorial Sloan-Kettering Cancer Center. For preparation of melanocyte cultures, connective tissue, deep dermis, and fat were dissected from skin, nevus, or choroid. Fragments of specimens were incubated overnight in Eagle's MEM with trypsin 0.25% at 4°C. Epidermis was separated from dermis in skin specimens, preparations were gently vortexed, and detached cells were grown in complete medium supplemented with 10 ng/ml PMA (Consolidated Midland Corp., Brewster, NY) and 10^{-8} M cholera toxin (Schwartz/Mann Biologicals, Orangeburg, NY) (melanocyte medium) (7), except where noted. Dermal cells were plated for nevus specimens and epidermal cells for cutaneous melanocyte cultures. For specific experiments, epidermal melanocytes were established in complete medium containing melanocyte growth factors derived from cell extracts of WI-38 fetal fibroblasts (McGF-W) or from the melanoma cell line SK-MEL-131 (McGF-M) (8).

Serological Assays. mAbs S6, S23, and S27 are IgG1 mouse mAbs reacting with different epitopes on ADAbp (5, 9, 10). mAbs were purified from ascites of hybridoma-bearing mice by 45% ammonium-sulfate precipitation followed by chromatography through DEAE-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Anti-mouse Ig mixed hemadsorption assay was performed as described previously (11). Indicator cells were prepared by conjugating purified rabbit anti-mouse γ heavy chain (Dako Corp., Santa Barbara, CA) to human O^+ red blood cells with 0.01% (wt/vol) chromium chloride. Assays were performed on cells grown in microtest plates (model 3040; Falcon Labware, Oxnard, CA). Target cells plated 2–3 d previously were incubated with serially diluted antibody for 1 h at room temperature. Target cells were washed and indicator cells were added for 45 min. Titers were defined as antibody dilution giving 20% rosetted target cells when evaluated by light microscopy. Indirect immunofluorescence assays were performed in Falcon Labware (model 3040) plates (12). Cells were fixed in 3.7% formaldehyde for 10 min, washed with PBS, and incubated with goat anti-mouse Ig conjugated to fluorescein (Cooper Biomedical, Inc., Malvern, PA) diluted 1/50 for 30 min. For absorption tests (13), mAb S23 or S27 was initially titered against FS10 target melanocytes and SK-RC-1 renal cancer cell lines, and a dilution that was two doubling dilutions below the titer endpoint was used for absorption. Diluted mAb was incubated with packed melanoma cells (1.5:1 vol/vol) or renal cancer cell line SK-RC-1 (positive control) for 1 h. Absorbing cells were centrifuged, and absorbed and unabsorbed mAb were tested for reactivity against FS10 melanocytes.

Immunohistological Staining. Frozen tissue sections 4–8 μm thick were cut using a Bright OTF cryostat with a 530 microtome (Hacker Instruments, Inc., Fairfield, NJ). For indirect immunofluorescence, cryostat-cut sections were fixed for 10 min with 1% formalin in PBS (pH 7.4). Tissue sections were washed, then incubated with primary antibody mAb S27 at 20 μg/ml for 1 h. All incubations were performed at room temperature in wet chambers.

Sections were washed and incubated for 45 min with secondary fluoresceinated antibody (1:40) (Cappel Laboratories, Cochranville, PA). Tissue sections were washed extensively in PBS and wet mounted in 90% glycerol in PBS. The specimens were examined with a fluorescence photomicroscope equipped with epifluorescence, using a 100 W mercury lamp (Nikon Inc., Garden City, NY). For indirect immunoperoxidase (14), frozen sections were treated for 10 min with 1% hydrogen peroxide in PBS to remove endogenous peroxidase activity, incubated with 10% normal goat serum for 20 min, then with 20 μg/ml mAb S27 overnight for 4°C. Secondary horseradish peroxidase–conjugated rabbit anti-mouse Ig (Tago Inc., Burlingame, CA) was incubated for 45 min. For the final reaction, diaminobenzidine 0.005% (wt/vol) (Sigma Chemical Co., St. Louis, MO) was

dissolved with 100 μl of 0.3% hydrogen peroxide. Frozen normal kidney tissue sections served as positive controls for each experiment. Negative controls included 20 μg/ml of mouse IgG1 mAb S1.

Radioimmunoprecipitation. Cell labeling, immunoprecipitation, and gel electrophoresis were carried out as previously described (15). Cells were labeled with [^{3}H]glucosamine (New England Nuclear, Boston, MA) over 72 h in glucose-free Eagle's MEM containing 7.5% dialyzed FCS, and cell extracts were prepared in NP-40 0.5% (vol/vol), 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl. Lysates were radioimmunoprecipitated with mAb followed by 5 ng/ml protein A–Sepharose (Pharmacia Fine Chemicals), then reduced with dithiothreitol (Boehringer-Mannheim, Indianapolis, IN); immunoprecipitates were resolved by SDS-PAGE.

Binding Assays with Radiolabeled Antibody and Adenosine Deaminase. Bovine intestine adenosine deaminase was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Adenosine deaminase was further purified by fast-performance liquid chromatography (Pharmacia Fine Chemicals) with a Mono Q anion exchange column. The column was eluted with a salt gradient from 5 mM to 1 M NaCl in MES buffer (2-[*N*-morpholino] ethane-sulfonic acid) (Sigma Chemical Co.), 0.05 M, pH 6. Purified adenosine deaminase gave a single band of ~38 kD on SDS-PAGE stained by Coomassie blue. Purified adenosine deaminase and purified mAbs S23 and S27 were labeled with ^{125}I (New England Nuclear) by the chloramine T method. Labeled protein was separated by a G50 fine Sephadex (Pharmacia Fine Chemicals) column. Radiolabeled adenosine deaminase was additionally dialyzed against PBS. The specific activity was 0.5–1.0 μCi/μg for adenosine deaminase (>80% binding by absorption tests with cultured melanocytes) and 1–5 μCi/μg for mAbs (>80% immunoreactive by absorption tests). SDS-PAGE revealed a single protein band of ^{125}I-labeled adenosine deaminase (~38 kD) and of mAbs (~160 kD) under nonreducing conditions. Binding assays and RIAs were performed by incubating 10^5 target cells with radiolabeled adenosine deaminase or mAb in the presence or absence of >10-fold excess cold adenosine deaminase or mAb, respectively, for 2 h at 4°C. Cells were pelleted for 30 s, washed, and counted for cell number and for radioactivity in a gamma counter. Specific binding was determined from the following calculation: Specific binding = (bound radiolabeled protein in the absence of cold protein) − (bound radiolabeled protein in the absence of excess cold protein). Nonspecific binding was <10% of total bound protein. Binding curves were determined by third order polynomial smoothing using Statgraphics software package (STSC, Inc., Rockville, MD).

Infection of Melanocytes with Murine Sarcoma Virus. Viral stocks were isolated from NIH 3T3 nonproducer clones infected with Kirsten murine sarcoma virus (Ki-MSV) or Harvey murine sarcoma virus (Ha-MSV) and superinfected with 4070A amphotrophic murine leukemia virus (MuLV) (16). Ki-MSV and Ha-MSV pseudotypes were harvested from 24-h cell-free supernatants and stored at −70°C. For infection, 10^5 melanocytes were treated for 60 min with DEAE/dextran (25 mg/ml), washed, and incubated with virus at a multiplicity of infection of 1 PFU/cell. Transforming activity of virus produced by infected melanocytes was determined by focus assays on NIH 3T3 cells (17). Focus forming units per milliliter of supernatant fluid were 3×10^4 for Ha-MSV and 3×10^3 for Ki-MSV (16). Cloning efficiency was determined by plating 10^5 cells in soft agar (0.75% bottom layer and 0.3% top layer) and scoring colonies of >50 cells at 2–3 wk.

In Vitro Transformation of Melanocytes. Two human newborn foreskin melanocyte cultures, FS1190 and FS10W, were transformed according to the following procedure. Before transformation, cell strains were maintained in melanocyte medium (7) as described above and were passaged at a regular interval of 3 weeks and at a ratio of 1:3. For induction of transformation, cell lines FS1190, passage 15, and FS10W, passage 29, were grown in complete medium containing PMA, cholera toxin and isobutylmethylxanthine (IBMX) 10^{-5} M (18). This supplemented medium induced rapid proliferation of melanocytes. Cultures were then passaged every 3 wk, and during the next 1–2 mo colonies of morphologically altered cells were observed. After 2 mo in PMA/cholera toxin/IBMX-supplemented medium, colonies were selected that grew in complete medium without PMA (PMA is the crucial mitogenic factor in melanocyte medium) (7). After initial

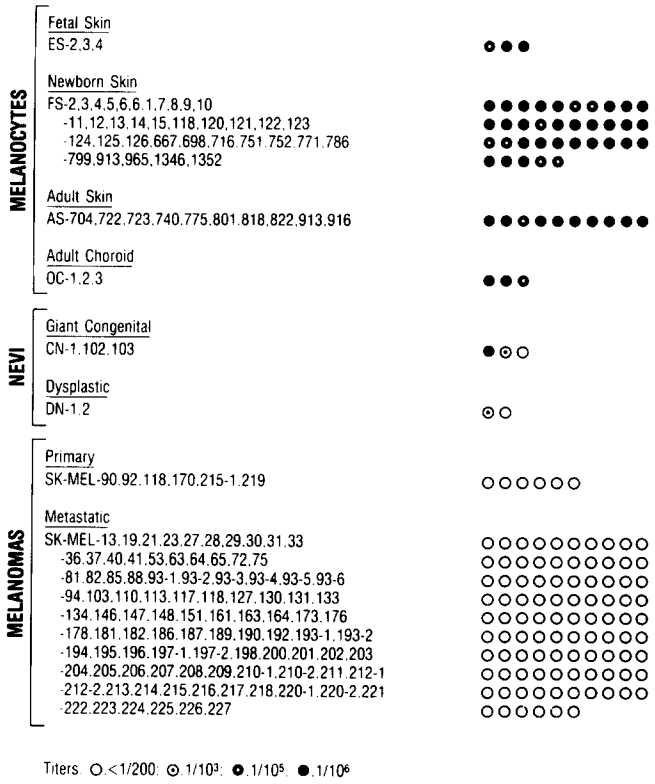

FIGURE 1. Serological typing for expression of ADAbp by mAb S27 with anti-mouse Ig mixed hemadsorption assays of cultured melanocytes (51 cell lines), nevi (5), and melanomas (102). Each circle represents a test with an individual cell line.

selection of transformed colonies, cells were grown in complete medium without any addition of PMA, cholera toxin, or IBMX. When parental cell lines FS1190 and FS10W were maintained in melanocyte medium in the absence of IBMX, no transformed colonies were observed.

Results

Adenosine Deaminase Binding Protein Is Expressed by Cultured Melanocytes but not by Melanomas. We have previously reported results of serological typing of cultured melanocytes and melanomas using a large panel of mouse mAbs (3, 4). Three categories of antigens have been distinguished: (*a*) antigens expressed by fetal, newborn, and adult melanocytes and by a subset of melanomas at intermediate or late stages of melanocyte differentiation (7 antigen systems); (*b*) antigens expressed by a subset of melanoma cell lines corresponding to early or intermediate stages of melanocyte differentiation (8 antigens); and (*c*) antigens widely expressed by cultured melanomas and melanocytes (82 antigens). We now describe a fourth category of melanocyte antigen, the ADAbp, that is expressed by cultured melanocytes but not by melanomas.

Fig. 1 shows the results of serological typing for expression of ADAbp on a panel of cultured melanocytes and melanomas using mAb S27. ADAbp was

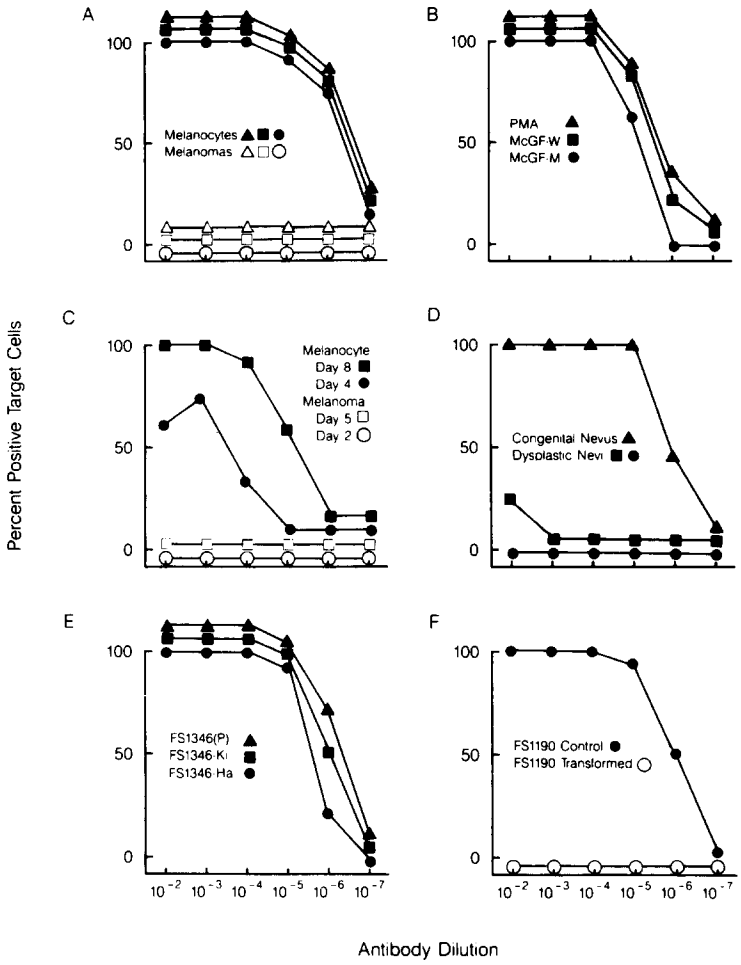

FIGURE 2. Serological typing for ADAbp expression by mAb S27 with anti-mouse Ig mixed hemadsorption assays. (*A*) Typing of three melanocyte (FS125, AS801, and OC1) and three melanoma (SK-MEL-28, SK-MEL-31, and SK-MEL-190) cell lines. (*B*) Typing of melanocyte cell line FS118 established in PMA or in melanocyte growth factors derived from WI-38 fetal fibroblast cell line (McGF-W), and cell line FS120 established in melanocyte growth factors derived from the melanoma cell line SK-MEL-131 (McGF-M) (see reference 8). (*C*) Typing of melanocytes and melanoma cells shortly after explantation into tissue culture; melanocyte culture FWS118 was typed 4 d after explantation and melanoma culture SK-MEL-146 was typed 2 d and 5 d after explantation. (*D*) Typing of melanocytic cells from a giant congenital nevus (CN-1) and dysplastic nevi (DN-1 and DN-2). (*E*) Typing of melanocyte cell line FS1346 infected with Ki-MSV (FS1346-Ki), Ha-MSV (FS1346-Ha), or uninfected cells [FS1346(P)]. (*F*) Typing of untransformed (FS1190 Control) and transformed (FS1190 Transformed) melanocyte cell line FS1190.

detected on the cell surface of 51 melanocyte cultures derived from fetal, newborn, and adult skin and adult choroid. Identical results were observed in assays using mAbs S6 and S23, which detect different epitopes on the ADAbp molecule. Fig. 2*A* demonstrates representative mixed hemadsorption assays for binding of mAb S27 to cultured melanocytes. In contrast to melanocytes, ADAbp was not detected on the cell surface of 102 melanoma cell lines derived from

either primary or metastatic lesions (Figs. 1 and 2*A*). Lack of binding of mAbs S23 and S27 by cultured melanomas was confirmed by binding assays with ^{125}I-labeled mAbs (see below). Absorption tests, a sensitive serological assay for cell surface antigen expression, failed to reveal any ADAbp expression by melanoma cells. Melanoma cell lines SK-MEL-23, SK-MEL-30, and SK-MEL-37 did not absorb any reactivity of mAbs S23 or S27 against the FS10 melanocyte cell line or against the renal carcinoma cell line SK-RC-1.

Melanoma cell lines corresponding to early, intermediate, and late stages of melanocyte differentiation, were ADAbp$^-$, whereas cultured melanocytes (at intermediate and late stages of differentiation) were uniformly ADAbp$^+$. Thus, stage of differentiation did not appear to influence the level of ADAbp expression by melanocytes.

Melanocytes, unlike cultured melanoma cells, require exogenous growth factors to proliferate in tissue culture. To demonstrate that ADAbp expression was not simply the result of induction by exogenous growth factors, melanocytes were established under different growth conditions and tested for expression of ADAbp (Fig. 2*B*). ADAbp was equally expressed by melanocytes established in: (*a*) the phorbol ester PMA with or without cholera toxin, (*b*) cholera toxin alone (these cultures contained a mixture of keratinocytes and melanocytes, but melanocytes were easily distinguished by their morphology and pigmentation), (*c*) melanocyte growth factor derived from the fetal fibroblast cell line WI-38, and (*d*) melanocyte growth factor derived from the melanoma cell line SK-MEL-131. Conversely, 18 melanoma cell lines grown in PMA with or without cholera toxin for periods of 1–3 mo remained ADAbp$^-$ (data not shown). Expression of ADAbp by melanocytes and lack of expression by melanomas were not related to rate of growth (doubling times ranged from 48 h to >120 h for melanocyte cultures and 20 h to >120 h for melanoma cultures) or degree of confluency in tissue culture. Melanocytes cultured in complete medium without growth factors stop proliferating within 5–7 d (7). No change in ADAbp expression, determined by titers of mAbs S6, S23, and S27, was observed 5 and 7 d after removal of the growth factors PMA and cholera toxin from melanocyte cultures, suggesting that ADAbp expression was not strictly related to cell proliferation.

ADAbp was detected on the cell surface of newborn foreskin melanocytes by mixed hemadsorption assays as early as 4 d after explantation into tissue culture (Fig. 2*C*). In these assays, pigmented, bipolar spindle-shaped melanocytes could easily be distinguished from other cell types, i.e., fibroblasts and keratinocytes, in the culture. Freshly cultured melanocytes, at day 4 after explant, were observed to express lower levels of ADAbp than cultures at day 8 (Fig. 2*C*). This low expression possibly is related to preparation of epidermal specimens in trypsin and EDTA for 18–24 h before placement in culture; cell surface ADAbp is removed by trypsin treatment (9). No ADAbp expression was detected in freshly explanted melanoma cultures as early as day 2 in culture (Fig. 2*C*).

ADAbp synthesis by melanocyte cultures was confirmed by radioimmunoprecipitation experiments using whole cell lysates of melanocytes metabolically labeled with [^{3}H]glucosamine (Fig. 3). The molecular size of ADAbp on melanocytes (120,000 mol wt) was identical to previously characterized ADAbp expressed by control renal cell carcinoma cell line SK-RC-1 (5, 9). ADAbp

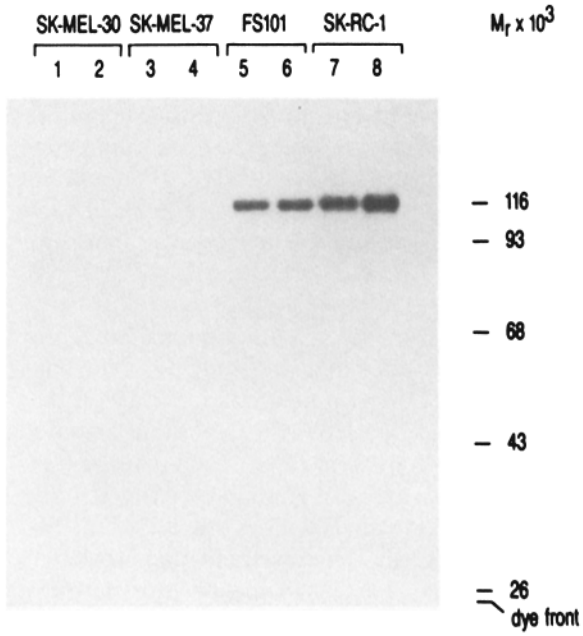

FIGURE 3. Immunoprecipitation of ADAbp from melanocyte cell line FS101, renal cell carcinoma SK-RC-1 (positive control) and melanomas SK-MEL-30 and SK-MEL-37. Cells were metabolically labeled with [^{3}H]glucosamine and cell lysates were immunoprecipitated with mAb S23 (lanes *1*, *3*, *5*, and *7*) or mAb S27 (lanes *2*, *4*, *6*, and *8*).

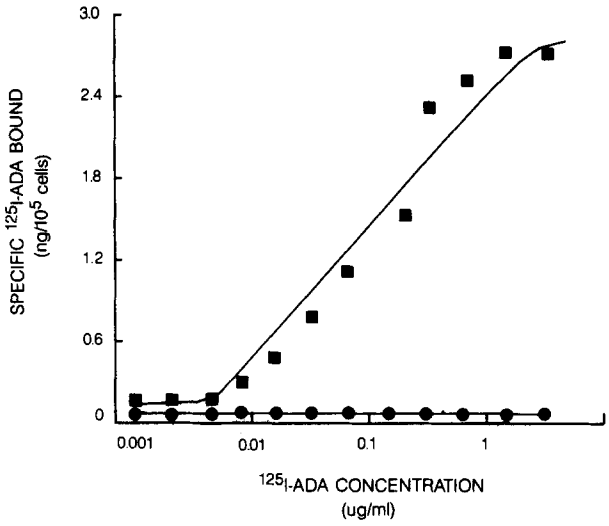

FIGURE 4. Specific binding of ^{125}I-labeled bovine adenosine deaminase per 10^5 cells to melanocyte cell line FS121 (■) and melanoma SK-MEL-23 (●).

expression was not detected in metabolically labeled melanoma cells even after extended exposure of autoradiographs (data not shown), demonstrating that undetectable cell surface expression by melanoma cells was not accompanied by discernible ADAbp in cytoplasmic compartments.

Studies with Radiolabeled Bovine Adenosine Deaminase and Anti-ADAbp mAbs. To confirm that ADAbp detected by serological techniques actually corresponded to binding sites for adenosine deaminase, ^{125}I-labeled bovine adenosine deaminase was tested for specific binding to cultured melanocytes and melanomas. Fig. 4 demonstrates an experiment showing saturable binding of

radiolabeled adenosine deaminase to the melanocyte cell line FS121 but no specific binding to the melanoma cell line SK-MEL-23. From this experiment it was estimated that FS121 melanocytes expressed a mean of 3.8×10^5 ADAbp molecules per cell. These calculations were based on one adenosine deaminase binding site per ADAbp molecule (19). In adenosine deaminase binding assays with three other newborn foreskin melanocyte cultures, FS101, FS123, and FS1404, 1.6×10^5, 3.6×10^5, and 9.2×10^4 mean binding sites per cell were detected, respectively. No specific binding of bovine adenosine deaminase was observed to the melanoma cell lines SK-MEL-13, SK-MEL-30, SK-MEL-31, SK-MEL-37, and SK-MEL-93.

Experiments with ^{125}I-labeled mAbs S23 and S27 gave a similar range for mean number of cell surface ADAbp molecules per melanocyte. Newborn foreskin melanocytes FS101, FS118, and FS120 had a mean of 1.4×10^5, 2.4×10^5, and 5.8×10^4 binding sites per cell, respectively. These calculations assumed that each ADAbp has one binding site for each mAb and that each divalent IgG mAb can bind to two ADAbp molecules at saturation. Radiolabeled mAbs S23 and S27 showed no specific binding to melanoma cell lines SK-MEL-23, SK-MEL-31, and SK-MEL-37. It has been previously demonstrated that mAb S27 does not inhibit binding of adenosine deaminase to ADAbp (5), and therefore inhibition experiments were not performed.

Cultured Congenital and Dysplastic Nevi Express Little or No Adenosine Deaminase Binding Protein. Nevi, which are benign collections of melanocytic cells in the skin, can contain morphologically normal or dysplastic-appearing cells. Dysplastic and giant congenital nevi are potentially premalignant lesions and have been observed to evolve into primary melanoma (2). Cultures of melanocytes derived from two dysplastic nevi and three giant congenital nevi were assayed for expression of ADAbp. Care was taken during preparation of nevus cultures to select dermal and epidermal areas within the specimen that contained nevus cells. Pigmented cells derived from giant congenital and dysplastic nevi expressed little or no ADAbp, with the exception of melanocytes derived from one congenital nevus specimen (Figs. 1 and 2*D*). The decrease or loss of ADAbp expression was the only phenotypic trait that distinguished pigmented cells of dysplastic and congenital nevi from cultured melanocytes derived from normal skin. Otherwise, cultures from congenital and dysplastic nevi and normal skin were phenotypically comparable: cells remained dependent on growth factors for proliferation and expressed intermediate and late markers of melanocyte differentiation but not early markers; notably, melanocytes cultured from dysplastic and congenital nevi did not express class II major histocompatibility (Ia) antigens or epidermal growth factor receptor, which are traits expressed by a subset of melanomas (4).

Immunohistological Studies with mAb S27 in Formaldehyde-fixed Frozen Tissues. A limited panel of formaldehyde-fixed, frozen sections of normal skin, junctional nevi, dysplastic nevi, and primary and metastatic melanomas was examined for staining with mAb S27 (20 μg/ml) by indirect immunofluorescence and immunoperoxidase techniques. (Fig. 5). There was detectable staining by mAb S27 of normal epidermal melanocytes in most specimens and of dermal fibroblasts in all specimens (Figs. 5 and 6). Staining of normal fibroblasts by

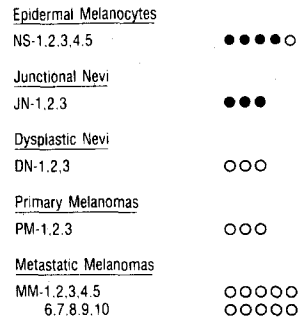

FIGURE 5. Typing for ADAbp expression with mAb S27 on frozen tissue sections by immunofluorescence and immunoperoxidase techniques. Tissues were fixed with 3.7% formaldehyde. (●) Positive staining; (○) no staining.

mAbs against ADAbp has been reported previously (10). No staining of epidermal melanocytes was observed in one specimen of palm skin (Fig. 5). mAb S27 stained pigmented cells in two junctional nevi with a heterogeneous pattern (Figs. 5 and 6). No staining of two dysplastic nevi or a small panel of primary and metastatic melanomas was detected (Figs. 5 and 6). It was observed that normal appearing melanocytes in the epidermis at the borders of dysplastic nevi, primary melanoma specimens, and cutaneous metastases were stained with mAb 27 (data not shown). These findings suggest that normal epidermal melanocytes in proximity to dysplastic nevi or primary melanomas have a normal phenotype and are not part of a field of premalignant or malignant cells.

Cultured Melanocytes Infected by Ha-MSV/Ki-MSV Continue to Express ADAbp. The relationship of malignant transformation and regulation of ADAbp on melanocytic cells was investigated further by infecting melanocytes with transforming amphotropic pseudotypes of Ha-MSV or Ki-MSV. Ha-MSV and Ki-MSV contain activated oncogenes of the *ras* family. In a previous report we have shown that infection of normal cultured human melanocytes with Ki-MSV or Ha-MSV, but not control amphotropic MuLV helper virus, led to induction of certain phenotypic traits of melanoma cells (16), including: (*a*) morphologic alterations characterized by clustering and piling up of cells, (*b*) increased growth rate and saturation density, (*c*) anchorage-independent growth with cloning frequencies in soft agar ranging from 0.04–0.1%, (*d*) induction of class II major histocompatibility (Ia) antigens, and (*e*) augmentation of cell surface expression of the disialoganglioside GD3 (16). Expression of Ia antigens and prominent expression of GD3 ganglioside are characteristics of melanoma cells. Despite these changes, melanocytes expressing activated *ras* remained dependent on exogenous growth factors for proliferation and were diploid at early passage (with some hyperdiploid cells at later passages), without any detectable chromosomal alterations or rearrangements. Infection with Ki-MSV and Ha-MSV did not induce any detectable change in the expression of ADAbp by melanocytes (16) (Fig. 2*E*). These results indicate that downregulation of ADAbp expression is not tightly regulated with other *ras*-induced transformation traits in melanocytes.

Loss of Expression of Adenosine Deaminase Binding Protein by Melanocytes Transformed In Vitro. Melanocytes and other normal human cells have been difficult to completely transform by a single oncogene as the transforming agent. Although activated *ras* expression in melanocytes was able to induce certain

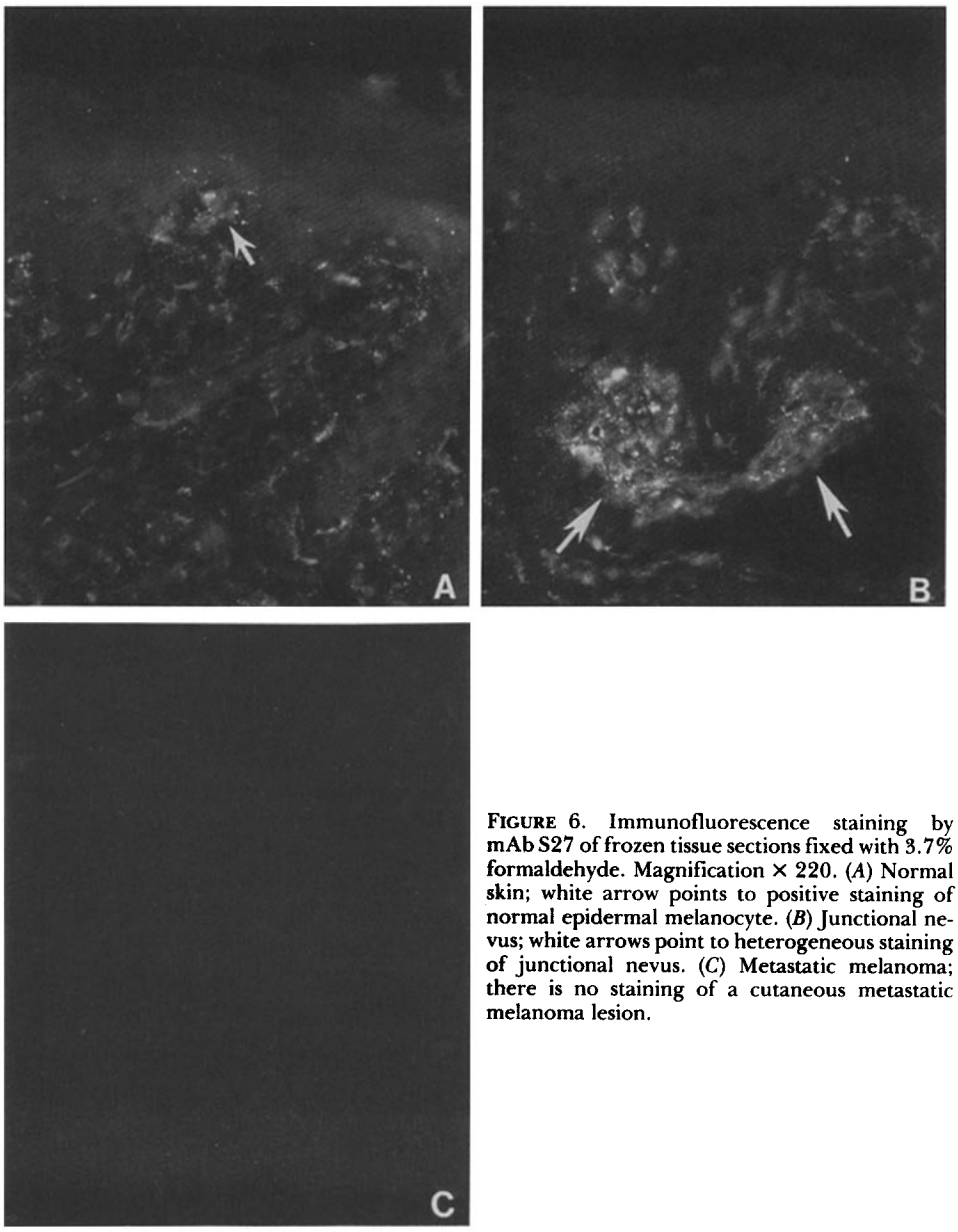

FIGURE 6. Immunofluorescence staining by mAb S27 of frozen tissue sections fixed with 3.7% formaldehyde. Magnification × 220. (*A*) Normal skin; white arrow points to positive staining of normal epidermal melanocyte. (*B*) Junctional nevus; white arrows point to heterogeneous staining of junctional nevus. (*C*) Metastatic melanoma; there is no staining of a cutaneous metastatic melanoma lesion.

characteristics of transformed cells (16), loss of ADAbp expression was not observed. During investigations of conditions that control growth of melanocytes, a novel system to transform human melanocytes was found (Eisinger, M. E., O. Marko, A. N. Houghton, manuscript in preparation). Transformed colonies were observed in rapidly dividing cultures of two independent melanocyte cell lines, FS1190 and FS10W, derived from newborn foreskin. To elicit rapidly proliferating cells, cultures were grown in PMA, cholera toxin, and IBMX (18), and transformed-appearing colonies appeared after 1–2 mo. Transformed colonies

had the following characteristics consistent with malignancy: (*a*) growth in complete medium without addition of exogenous growth factors, (*b*) spindle and polygonal-shaped cells with nuclear atypia and a high nuclear/cytoplasmic ratio, (*c*) high saturation density, (*d*) formation of colonies in soft agar, and (*e*) aneuploid karyotype. On the other hand, the parental newborn foreskin melanocyte cell lines FS1190 and FS10W were dependent on exogenous factors for growth, had a dendritic, bipolar-spindle morphology, grew at a low saturation density, were unable to grow in soft agar, and had a diploid karyotype. Transformation of FS1190 and FS10W cells was associated with loss of detectable ADAbp expression. Parental FS1190 and FS10W cultures expressed levels of ADAbp that were typical of other melanocyte cultures but transformants of FS1190 and FS10W were ADAbp$^-$ (Fig. 2*F*). This transformation system confirms that ADAbp expression can be regulated during the process of melanocyte transformation.

Discussion

A constellation of phenotypic traits distinguishes transformed cells from their normal cell counterparts. Malignant cells characteristically acquire an altered morphology and appearance, grow in an anchorage-independent fashion, require lower amounts of exogenous growth factors, and have an abnormal karyotype. Despite these profound alterations, analysis of tumor cells has demonstrated that malignant transformation generates mainly quantitative changes in expression of cell components (i.e., increase or decrease) or modification of existing components (e.g., posttranslational modification) rather than qualitative changes (i.e., presence or absence) (20). The subtle nature of critical phenotypic alterations is demonstrated in certain cell systems where regulation of a single protein can lead to transformation (21, 22). In our studies of determinants detected by mAbs S6, S23, and S27, ADAbp was strongly expressed on melanocytes but was not detected on melanoma cells. These findings suggest that expression of ADAbp may differ qualitatively between melanocytes and melanoma cells. However, this presumption is limited by the sensitivity and specificity of the assays used to measure ADAbp, and therefore it is possible that small numbers of ADAbp molecules on melanoma cells are not detected or that only some determinants on the ADAbp molecule are regulated during transformation.

Lack of staining of melanocytes in occasional skin specimens could be related to an ADAbp$^-$ phenotype (for instance in palm skin that may contain hyperproliferative epidermal cells) or to insufficient sensitivity of immunostaining techniques. In tests against cultured melanocytes fixed with 3.7% formaldehyde, mAb S27 gave only weak to moderate staining by indirect immunofluorescence techniques (titers of 1/200 to 1/3,200), indicating that immunostaining techniques were not nearly as sensitive as mixed hemadsorption assays (titers $1/10^5$ to $1/10^6$) to detect ADAbp expression. In addition, epidermal melanocytes can be difficult to evaluate by immunostaining methods because they comprise only a small proportion of cells in the basal layer of the epidermis, they have endogenous peroxidase activity that can interfere with peroxidase-mediated staining, and they have pigment that can quench immunofluorescence staining.

Clark and coworkers (1) have proposed five "lesional steps" in the progression from melanocyte to melanoma: (*a*) pigmented cells that are regulated and

restrained by a differentiation program, (*b*) dysplastic pigmented cells, (*c*) primary melanoma cells without competence for metastases, (*d*) primary melanoma cells with competence for metastases, and (*e*) metastatic melanoma cells. In relation to this scheme, normal melanocytes were ADAbp$^+$, dysplastic nevi expressed little or no ADAbp, and primary and metastatic melanoma cells were ADAbp$^-$. These data suggest that ADAbp expression decreases at a relatively early stage in the pathogenesis of melanoma, coinciding with the appearance of cytologic atypic (dysplastic nevi) and before acquisition of autonomous growth and invasiveness (primary melanoma). Studies of melanocyte transformation using culture systems also suggest an association between genetic alterations and ADAbp expression. Analysis of cultured melanocyte cells that corresponded to presumed early stages of transformation (e.g., melanocytes expressing activated *ras* genes and cells derived from dysplastic nevi) showed that downregulation of ADAbp on melanocytes is not coregulated with certain growth-related traits of malignant melanocytes, specifically anchorage-independent growth or growth factor–independent proliferation. One possibility is that chromosomal instability is closely linked to loss of ADAbp expression, particularly since karyotypic abnormalities can be observed in some melanocytes from dysplastic nevi and in most primary and metastatic melanoma cells (23, 24). Further in vitro studies to dissect stages of melanocyte transformation will be necessary to test this possibility.

Other markers have been proposed to distinguish "lesional steps." Certain phenotypic traits of melanocyte cells derived from normal skin, nevi, primary melanoma, and metastases have been examined in detail by Herlyn et al. (25, 26) according to Clark's model of tumor progression (1). Substantial alteration in ganglioside pattern and changes in expression of several antigens have been found that distinguish melanocytes derived from different lesional steps. Immunohistological techniques also have been used to describe a number of antigens that distinguish melanomas from benign nevi (27–32). According to serological specificity and molecular size, none of these antigens appear to correspond to ADAbp.

ADAbp is expressed by other cell lineages in addition to melanocytes (10, 33, 34). A relationship between ADAbp expression and malignancy was first suggested in studies evaluating isoenzyme expression in normal and malignant tissues (35). Decreases in ADAbp expression have been described in certain epithelial cancers including colon and kidney carcinomas, and in virally transformed fibroblasts (36–38). In the case of colon carcinomas, more detailed studies have demonstrated both increased and decreased ADAbp expression in tumors compared to normal colon mucosa (39–40). Immunohistological studies have shown a correlation between increased ADAbp expression and differentiation state of the tumor, suggesting that ADAbp is regulated as part of the differentiation program of normal colon epithelium (39–40). However, within the melanocyte lineage there was no apparent association between ADAbp expression and stage of differentiation, consistent with the notion that ADAbp is expressed in the normal melanocyte lineage but is specifically downregulated as part of a transformation process. Expression of ADAbp can be contrasted with another set of cell surface glycoproteins, Ia antigens, that are regulated by both transformation and differentiation in melanocytes (4). We have found that Ia can be induced in

melanocytes by viral *ras* gene products (16) and by melanocyte transformation in vitro (Eisinger, M., L. J. Davis, and A. N. Houghton, unpublished observation) and additionally that Ia can be regulated in cloned melanoma cell lines as part of the melanocyte differentiation program (4).

Although ADAbp is known to bind adenosine deaminase (19), the function of the binding protein itself is unknown. Adenosine deaminase catalyzes irreversible deamination of adenosine to inosine and deoxyadenosine to deoxyinosine, and binding to ADAbp does not seem to affect the activity of the enzyme (19, 33). Unlike many cell surface receptors, evidence suggests that ADAbp does not transport adenosine deaminase into cells but rather holds the enzyme at the cell surface (41). One possibility is that loss of ADAbp could allow transformed melanocytes to become susceptible to inductive effects of adenosine. Studies to evaluate the possible role of adenosine on melanocytes' growth and differentiation are underway. ADAbp could influence intracellular events, for instance DNA replication or repair through effects on purine metabolism. According to either of these possibilities, the loss of expression of ADAbp by melanoma cells would directly influence the transformed phenotype, perhaps through regulation of cell growth or error-prone synthesis and repair of DNA. On the other hand, loss of ADAbp expression may be a secondary event, and therefore a consequence of changes in growth or differentiation of malignant cells. In either case, well-defined markers that distinguish melanocytes from melanoma cells will facilitate investigations of melanocyte transformation in vitro and in vivo.

Summary

It has been proposed that the pathogenesis of melanoma proceeds through multiple stages, ranging from benign proliferation of melanocytic cells to acquisition of the capacity to invade tissues and metastasize. During investigations of cell surface antigens expressed by melanocytes and melanoma, we identified an antigen system that was expressed by cultured normal melanocytes but not by melanoma cell lines. mAbs against this antigen detected a 120-kD cell surface glycoprotein on melanocytes. This molecule had been identified previously as the binding protein for adenosine deaminase (ADAbp). ADAbp was expressed by 51 melanocyte cell lines derived from normal fetal, newborn, and adult skin and adult choroid, but not by 102 melanoma cell lines derived from primary and metastatic lesions. Studies with radiolabeled bovine adenosine deaminase confirmed that melanocytes expressed binding sites for adenosine deaminase, but no binding sites were detected on cultured melanoma cells. Further studies showed that $ADAbp^+$ melanocytes became $ADAbp^-$ upon malignant transformation in vitro. Immunohistochemical studies on a panel of frozen tissues demonstrated reactivity of anti-ADAbp mAbs with epidermal melanocytes and benign junctional nevi, but not with potentially premalignant dysplastic nevi or primary/metastatic melanoma lesions. These studies demonstrate that ADAbp expression is lost with malignant transformation of melanocytes, presumably at an early stage in the transformation process.

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References

1. Clark, W. H., Jr., D. E. Elder, D. Guerry, IV, M. N. Epstein, M. H. Greene, and M. Van Horn. 1984. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Hum. Pathol.* 15:1147.
2. National Institutes of Health Consensus Development Conference Summary. 1983. Precursors to Malignant Melanoma. 4:1–10.
3. Houghton, A. N., M. Eisinger, A. P. Albino, J. G. Cairncross, and L. J. Old. 1982. Surface antigens of melanocytes and melanoma: markers of melanocyte differentiation and melanoma subsets. *J. Exp. Med.* 156:1755.
4. Houghton, A. N., F. X. Real, L. J. Davis, C. Cordon-Cardo, and L. J. Old. 1987. Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. *J. Exp. Med.* 164:812.
5. Andy, R. J., C. L. Finstad, L. J. Old, K. O. Lloyd, and R. Kornfeld. 1984. The antigen identified by a mouse monoclonal antibody raised against human renal cancer cells is the adenosine deaminase binding protein. *J. Biol. Chem.* 259:12844.
6. Carey, T. E., T. Takahashi, L. A. Resnick, H. F. Oettgen, and L. J. Old. 1967. Cell surface antigens of human malignant melanoma. I. Mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proc. Natl. Acad. Sci. USA.* 73:3278.
7. Eisinger, M., and O. Marko. 1982. Selective proliferation of normal human melanocytes *in vitro* in the presence of phorbol ester and cholera toxin. *Proc. Natl. Acad. Sci. USA.* 79:2018.
8. Eisinger, M. E., O. Marko, S.-I. Ogata, and L. J. Old. 1985. Growth regulation of human melanocytes. Mitogenic factors in extracts of melanoma, astrocytoma, and fibroblast cell lines. *Science (Wash. DC).* 229:984.
9. Ueda, R., S.-I. Ogata, D. M. Morrissey, C. L. Finstad, J. Szkudlarek, W. F. Whitmore, H. F. Oettgen, K. O. Lloyd, and L. J. Old. 1981. Cell surface antigens of human renal cancer defined by mouse monoclonal antibodies, identification of tissue-specific kidney glycoproteins. *Proc. Natl. Acad. Sci. USA.* 78:5122.
10. Finstad, C. F., C. Cordon-Cardo, N. H. Bander, W. F. Whitmore, M. R. Melamed, and L. J. Old. 1985. Specificity analysis of mouse monoclonal antibodies defining cell surface antigens of human renal cancer. *Proc. Natl. Acad. Sci. USA.* 82:2955.
11. Dippold, W. G., K. O. Lloyd, L. T. C. Li, H. Ikeda, H. F. Oettgen, and L. J. Old. 1980. Cell surface antigens of human malignant melanoma. Definition of six new antigenic systems with mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 77:6114.
12. Houghton, A. N., H. Brooks, R. J. Cote, M. C. Taormina, H. F. Oettgen, and L. J. Old. 1983. Detection of cell surface and intracellular antigens by human monoclonal antibodies: hybrid cell lines derived from lymphocytes of patients with malignant melanoma. *J. Exp. Med.* 158:53.
13. Houghton, A. N., M. C. Taormino, H. Ikeda, T. Watanabe, H. F. Oettgen, and L. J. Old. 1980. Serological survey of normal humans for natural antibody to cell surface antigens of melanoma. *Proc. Natl. Acad. Sci. USA.* 77:4260.
14. Cordon-Cardo, C., C. L. Finstad, N. H. Bander, and M. R. Melamed. 1987. Immunoanatomic distribution of cytostructural and tissue-associated antigens in the human urinary tract. *Am. J. Pathol.* 126:269.

15. Houghton, A. N., T. M. Thomson, D. Gross, H. F. Oettgen, and L. J. Old. 1984. Surface antigens of melanoma and melanocytes. Specificity of induction of Ia antigens by human gamma interferon. *J. Exp. Med.* 160:255.
16. Albino, A. P., A. N. Houghton, M. Eisinger, J. S. Lee, R. R. S. Kantor, A. I. Oliff, and L. J. Old. 1986. Class II histocompatability antigen expression in human melanocytes transformed by Harvey murine sarcoma virus (Ha-MSV) and Kirsten MSV retroviruses. *J. Exp. Med.* 164:1710.
17. Pincus, T., J. W. Hartley, and W. P. Rowe. 1975. A major genetic locus affecting resistance to infection with murine leukemia virus. IV. Dose-response relationships in Fv-1 sensitive and resistant cell cultures. *Virology.* 65:333.
18. Halaban, R., and F. D. Alfano. 1984. Selective elimination of fibroblasts from cultures of normal human melanocytes. *In Vitro.* 20:447.
19. Daddona, P. E., and W. N. Kelley. 1979. Human adenosine deaminase. Stoichiometry of the adenosine deaminase-binding protein complex. *Biochim. Biophys. Acta.* 580:302.
20. Celis, J. E., R. Bravo, P. Mose Larsen, S. J. Fey, J. Bellatin, and A. Celis. 1983. Expression of cellular proteins in normal and transformed human cultured cells and tumors. Two-dimensional gel electrophoresis as a tool to study neoplastic transformation and cancer. *In* Two Dimensional Gel Electrophoresis of Proteins: Methods and Applications. J. E. Celis and R. Bravo, editors. Academic Press, New York. 307–342.
21. Bishop, J. M. 1987. The molecular genetics of cancer. *Science (Wash. DC).* 235:305.
22. Bishop, J. M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* 52:301.
23. Balaban, G. B., M. Herlyn, W. H. Clark, Jr., and P. C. Nowell. 1986. Karyotypic evolution in human malignant melanoma. *Cancer Genet. Cytogenet.* 19:113.
24. Pederson, M. I., J. W. Bennett, and N. Wang. 1986. Nonrandom chromosomal structural aberrations and oncogene loci in human malignant melanoma. *Cancer Genet. Cytogenet.* 20:11.
25. Herlyn, M., J. Thurin, G. Balaban, J. L. Bennicelli, D. Herlyn, D. E. Elder, E. Bondi, D. Guerry, P. Nowell, W. E. Clark, and H. Koprowski. 1985. Characteristics of cultured human melanocytes isolated from different stages of tumor progression. *Cancer Res.* 45:5670.
26. Herlyn, M., G. Balaban, J. Bennicelli, D. Guerry, IV, R. Halaban, D. Herlyn, D. E. Elder, G. G. Maul, Z. Steplewski, P. C. Nowell, W. H. Clark, and H. Koprowski. 1985. Primary melanoma cells of the vertical growth phase: similarities to metastatic cells. *J. Natl. Cancer Inst.* 71:283.
27. Johnson, J. P., M. Demmer-Dieckmann, T. Meo, M. R. Hadam, and G. Riethmuller. 1981. Surface antigens of human melanoma defined by monoclonal antibodies. I. Biochemical characterizations of two antigens found on cell lines and fresh tumors of diverse origin. *Eur. J. Immunol.* 11:825.
28. Holzman, B., J. P. Johnson, P. Kaudewitz, and G. Riethmuller. 1985. *In situ* analysis of antigens on malignant and benign cells of the melanocyte lineage. Differential expression of two cell surface molecules gp75 and p89. *J. Exp. Med.* 161:366.
29. Lehman, J. M., B. Holzmann, E. W. Brietbart, P. Schmiegelow, G. Riethmuller, and J. P. Johnson. 1987. Discrimination between benign and malignant cells of the melanocyte lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. *Cancer Res.* 47:841.
30. Brocker, E. B., L. Suter, J. Bruggen, D. J. Ruiter, E. Macher, and C. Sorg. 1985. Phenotypic dynamics in human malignant melanoma. *Int. J. Cancer.* 36:29.
31. Ruiter, D. J., G. M. Dingjan, P. M. Steijlen, M. van Beveren-Hooyer, C. B. Graaff-Reitsma, W. Bergmann, G. N. P. van Mujen, and S. O. Warnaar. 1985. Monoclonal

antibodies selected to discriminate between malignant melanomas and nevocellular nevi. *J. Invest. Dermatol.* 85:4.

32. Holzman, B., E. B. Brocker, J. M. Lehmann, D. J. Ruiter, C. Sorg, G. Riethmuller, and J. P. Johnson. 1987. Tumor progression in human malignant melanoma: five stages defined by their antigenic phenotype. *Int. J. Cancer.* 39:466.
33. Van der Weyden, M. B., and W. N. Kelly. 1976. Human adenosine deaminase. Distribution and properties. *J. Biol. Chem.* 251:5448.
34. Schrader, W. P., and A. R. Stacy. 1979. Immunoassay of the adenosine deaminase complexing proteins of human tissues and body fluids. *J. Biol. Chem.* 254:11958.
35. Trotta, P. P., and M. E. Balis. 1978. Characterization of adenosine deaminase from normal colon and colon tumors. Evidence for tumor-specific variants. *Biochemistry.* 17:270.
36. Inwood, M., S. Povey, and J. D. A. Delhanty. 1980. Comparison of isozymes in fetal, adult and transformed fibroblasts. *Cell Biol. Int. Rep.* 4:327.
37. Herbschleb-Voogt, E., J. Ten Kate, and P. Meera Khan. 1983. Adenosine deaminase complexing protein (ADCP): a transformation sensitive protein with potentials of a cancer marker. *Anticancer Res.* 3:95.
38. Ten Kate, J., J. T. Wijnen, J. Boldewijn, P. M. Meera Khan, and F. T. Bosman. 1985. Immunohistochemical localization of adenosine deaminase complexing protein in intestinal mucosa and in colorectal adenocarcinoma as a marker for tumour cell heterogeneity. *Histochem. J.* 17:23.
39. Ten Kate, J., J. T. Wijnen, R. G. M. van der Goes, R. Quadt, G. Griffioen, F. T. Bosman, and P. Meera Khan. 1984. Quantitative changes in adenosine deaminase isozymes in human colorectal adenocarcinomas. *Cancer Res.* 44:4688.
40. Ten Kate, J., H. F. G. M. van den Ingh, P. Meera Khan, and F. T. Bosman. 1986. Adenosine deaminase complexing protein (ADCP) immunoreactivity in colorectal adenocarcinoma. *Int. J. Cancer.* 37:479.
41. Andy, R. J., and R. Kornfeld. 1982. The adenosine deaminase binding protein of human skin fibroblasts is located on the cell surface. *J. Biol. Chem.* 257:7922.