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Journal
Journal of the National Cancer Institute, 84(3)

ISSN
0027-8874

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Publication Date
1992-02-05

DOI
10.1093/jnci/84.3.165

ARTICLES

Coexpression of Vimentin and Keratins by Human Melanoma Tumor Cells: Correlation With Invasive and Metastatic Potential

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Background: **Several protein markers, including vimentin, have been used to diagnose human melanoma. Because melanoma often has metastasized by the time of diagnosis, early markers prognostic for metastatic potential need to be identified. Commonly, vimentin is found in mesenchymal cells, and keratins are present in epithelial cells, but recent studies report coexpression of vimentin and keratin(s) in epithelial and nonepithelial neoplasms, including some melanomas.** ***Purpose:*** **Our purpose was to determine whether coexpression of vimentin and keratin(s) is correlated with tumor cell invasion and metastatic behavior.** ***Methods:*** **We evaluated nine human melanoma cell lines expressing vimentin and other markers of aggressive tumor behavior (HMB-45, S-100, HLA-ABC class I and HLA-DR class II histocompatibility antigens, and K8 and K18 keratins). Levels of K8 and K18 keratins were determined in the highly metastatic C8161 cell line, the poorly metastatic A375P line, and the moderately metastatic A375M line. To determine whether the presence of keratin affects migratory ability, we altered the conformational structure of keratin filaments in C8161 cells by transfection with a mutant K18 complementary DNA. We also determined messenger RNA levels of human type IV collagenase, an enzyme marker for invasion and metastasis.** ***Results:*** **In A375P cells, two-dimensional electrophoresis with Coomassie-stained gels, immunoblotting, and immunofluorescence staining showed no detectable levels of K8 or K18. A375M cells showed low levels of K8 and K18 by Western and Northern blotting, with a distinctive fluorescent subpopulation of cells. In comparison, K8 and K18 levels in C8161 cells were high in all cells. Type IV collagenase messenger RNA levels were lowest in A375P cells and highest in C8161 cells, correlating with invasive ability in vitro and metastatic potential in athymic nude mice. The transfectant clones C1070-10 and C1070-14 derived from the C8161 parent line showed dramatic morphological changes, disrupted keratin filaments, and decreased invasive and metastatic potential directly correlated with a reduction in migratory activity.** ***Conclusion:*** **These findings show a correlation between the coexpression of vimentin with K8 and K18 keratins and the invasive and metastatic behavior of three representative human melanoma cell lines.** **[J Natl Cancer Inst 84:165–174, 1992]**

Received June 17, 1991; revised September 16, 1991; accepted November 19, 1991.

Supported by Public Health Service grant CA-54984 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; by the Arizona Disease Control Research Commission; and by the Eric Staniek Family.

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We thank Dr. C. George Ray for the editorial review, Dr. Jeffrey M. Trent and Floyd H. Thompson for performing the karyotypic analyses, Ms. Virginia Clark for her scientific expertise, Dr. Gloria Heppner for use of the Foundation Nude Mouse Facility, and Dr. Robert G. Oshima for the gift of the LK442-K18-1070 plasmid.

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A major problem in cancer management is metastasis—the migration of tumor cells in the vasculature and tissue parenchyma that gives rise to tumors at multiple locations in the body. Of the many types of cancers, malignant melanoma has been steadily increasing in incidence in the United States for several decades. It is estimated that, by the year 2000, one in 90 Americans will be afflicted with this disease (*1*). This incidence has been attributed to both atmospheric ozone degradation and the increasing aesthetic value placed on a suntan by Western populations. Whatever the societal and environmental contributions to the etiology of melanoma may be, alarmingly little is known about the fundamental biology of its aggressiveness in some individuals and quiescence in others.

From a clinical point of view, early diagnosis of melanoma determines disease prognosis. Unfortunately, there is good evidence to suggest that, at the time of diagnosis, the melanoma may have already metastasized (*2*), depending on a variety of factors such as tumor level and thickness (*3*). Therefore, specific markers prognostic for metastatic potential need to be identified.

The ability to invade or penetrate biological matrices is of primary importance in the development of malignant melanoma as well as the genesis of many kinds of advanced malignancy, because these matrices are encountered by tumor cells during intravasation, extravasation, and dissemination. A three-step hypothesis describing the sequence of biological events involved in tumor-cell interaction with such extracellular matrices has been proposed (*4,5*): (*a*) tumor cell attachment to a matrix substratum, (*b*) local degradation of the matrix by tumor cell-secreted proteases, and (*c*) tumor cell locomotion into the matrix modified by proteolysis. One enzyme marker shown to be closely associated with invasive and metastatic potential is type IV collagenase, which specifically degrades type IV collagen, the major structural component of basement membranes (*4-10*).

Classical diagnosis of human melanoma has relied on the presence of several protein markers, including HMB-45 and S-100 antigens and vimentin (*11-17*). Vimentin and the keratins are intermediate filaments, which are the principal components of the cytoskeleton of mammalian cells [*see* review in (*18-20*)]. Vimentin is characteristically found in mesenchymal cells, whereas keratins are usually found in epithelial cells. Although earlier studies emphasized the use of intermediate filaments as cell type-specific markers in differentiation and pathology (*21,22*), recent reports have confounded the literature with numerous demonstrations of the coexpression of vimentin and keratin(s) in epithelial and nonepithelial neoplasms, including some melanomas (*23-26*). The purpose of our study was to extend these observations by measuring specific markers in several human melanoma cell lines of differing metastatic potentials and to determine whether a correlation exists between the coexpression of vimentin and keratin(s) and tumor cell invasive and metastatic properties.

Materials and Methods

Human Melanoma Cell Lines

A375P, a poorly metastatic variant, and A375M, an intermediate metastatic variant (*27*), were developed from the human melanotic A375 parental cell line. These cell lines were supplied by I. J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston). The C8161 cell line was established from an amelanotic metastasis from a patient with recurrent, metastasizing malignant melanoma (*28*). Cell lines UACC-456, UACC-383, UACC-608, and UACC-827 were established at the University of Arizona Cancer Center Core Facility from patients with metastatic melanoma. Cell lines BOWES and HS294 were purchased from the American Type Culture Collection, Rockville, Md., and have been well characterized (*29,30*). All cell lines were stored as frozen stocks. They were maintained in culture for no longer than 2-3 weeks for each set of experiments to ensure that their metastatic phenotype would not change as a result of prolonged passage in vitro.

Cells were grown as adherent cultures in tissue culture flasks (Falcon Plastics, Lawndale, Calif.) containing Dulbecco's modified Eagle medium (DMEM) (Irvine Scientific, Santa Ana, Calif.). DMEM was supplemented with a 10% (1:1, vol/vol) mixture of heat-inactivated fetal bovine serum (FBS)-calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 0.1% gentamicin sulfate (GIBCO Laboratories). The flasks were incubated at 37 °C in a humidified incubator in 5% CO_2 and 95% air. Cells were detached by a 10-minute incubation in 2 m*M* EDTA in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) at pH 7.4, and a single-cell suspension was prepared by trituration. Cell numbers were determined by using a hemacytometer, and all cultures were found to be free of *Mycoplasma* spp. contamination with the Gen-Probe Rapid Detection System (Fisher Scientific, Tustin, Calif.).

Transfection of C8161 Cells

Transfection of C8161 cells was performed with the plasmid LK442-K18-1070, which contains a deleted human K18 complementary DNA (cDNA) under the control of a human β-actin promoter (*31*) and produces disrupted keratin filaments. This plasmid also carries the gene that codes for *Escherichia coli* xanthine-guanine phosphoribosyltransferase (gpt) as a drug-selection marker. The plasmid pHβApr-1-neo (*32*), which was obtained from J. Leavitt (Linus Pauling Institute of Science and Medicine, Palo Alto, Calif.), harbors a gene for resistance to the antibiotic G418 and was used for a control transfection (C-NEO cells). The plasmids were introduced into C8161 cells using the calcium phosphate precipitation method (*33,34*), and positive colonies were selected according to their ability to grow in DMEM containing 25 μg/mL mycophenolic acid, 2 μg/mL aminopterin, 15 μg/mL hypoxanthine, 250 μg/mL xanthine, 10 μg/mL thymidine, and 25 μg/mL adenine (Sigma Chemical Co., St. Louis, Mo.) for the gpt phenotype or 0.8 μg/mL G418 (GIBCO Laboratories) for the control neo phenotype.

Chromosomal Analysis

G- and Q-banding analyses of all cell lines were performed under routine conditions as previously described (*27,35*) to verify that the cell lines used were of human origin and displayed the characteristics of melanoma.

Analysis of Vimentin and Keratins

As described below, the intermediate filaments were identified by two-dimensional gel electrophoresis and Western blot

analysis, by immunofluorescence microscopy, and by Northern blot analysis.

Two-dimensional gel electrophoresis and Western blot analysis. The first-dimension separation of proteins was performed using nonequilibrium pH gradient gel electrophoresis (*36*) in tube gels (containing 2% ampholines; pH range, 3.5-9.5; Bio-Rad Laboratories, Richmond, Calif.) at 3700 V/h. The second dimension was run on 12.5% polyacrylamide gels containing sodium dodecyl sulfate (*37*), which were either stained with Coomassie BBR-250 (Bio-Rad Laboratories; 0.25% Coomassie BBR-250 in 25% isopropanol-10% acetic acid) or transblotted onto a sheet of Immobilon-P membrane (Millipore Corp., Bedford, Mass.). Visualization of the primary antibody was facilitated using a horseradish peroxidase-conjugated secondary antibody and 4-chloro-1-naphthol (Bio-Rad Laboratories).

Immunofluorescence microscopy. Indirect immunofluorescence microscopy was performed on all three cell lines, which were grown on glass coverslips to approximately 70% confluence. For the detection of vimentin, the V9 mouse monoclonal antibody against vimentin (DAKO Corp., Santa Barbara, Calif.) was diluted 1:50 in CMF-PBS. This antibody has been characterized previously (*24*). Monoclonal antibody 10.11 (*25*), which recognizes K8 and K18 keratins, was diluted 1:3000 in CMF-PBS.

All cell cultures, which were on coverslips, were rinsed in CMF-PBS three times. They were then immersed in methanol for 5 minutes, frozen, treated with acetone for 3 minutes, and then rinsed in CMF-PBS, after which they were incubated with the appropriate primary and secondary antibodies. For controls, the primary antibody was omitted, and the cells were incubated with the secondary antibody alone, in addition to incubation with normal mouse IgG followed by the appropriate rhodamine- or fluorescein-conjugated secondary antibody.

For detection of S-100 and HMB-45 antigens and histocompatibility antigens HLA-ABC and HLA-DR, which are used in detection of pathological disease, cells were prepared as described above and stained with the following antibodies using indirect immunofluorescence microscopy: rabbit antibody to bovine S-100 antigen (DAKO Corp.) (*38*), mouse antibody to human HMB-45 antigen (ENZO Diagnostics, Inc., New York, N.Y.) (*39*), mouse antibody to human HLA-DR antigen (Becton Dickinson, San Jose, Calif.), and mouse antibody to human HLA-ABC antigen. Photographs were taken with a Zeiss standard 18 fluorescence microscope with automatic rhodamine and fluorescein filter sets.

Northern blot analysis. Total RNA was extracted from the cells by the guanidine thiocyanate-cesium chloride method (*40*). The RNA was poly A selected and separated by gel electrophoresis in 1% agarose containing 2.2 *M* formaldehyde (*41*). RNA marker lanes were subsequently excised and stained with ethidium bromide. The RNA gels were capillary blotted onto 0.1-μm nylon membranes (GeneScreen Plus; DuPont, Wilmington, Del.), baked, and hybridized with the following nick-translated DNA probes: the pH3A plasmid for human type IV collagenase (*42*), the hp4F1 probe for vimentin (*43*), and the pK 811 and pK 189 probes for K8 and K18, respectively (*31*). These blots were reprobed for β-actin to ensure equal loading. The relative amount of each message was determined densitometrically using a model 620 video densitometer and a one-dimensional computer analysis program (Bio-Rad Laboratories).

Tumor Cell Invasion Assay

The Membrane Invasion Culture System (MICS) assay was used to evaluate the degree of tumor cell invasion through a reconstituted basement membrane (Matrigel; Collaborative Research, Inc., Bedford, Mass.) in vitro as previously described (*44*). Matrigel is composed of laminin, type IV collagen, entactin, and heparan sulfate proteoglycans (*45*). Briefly, 5×10^4 tumor cells were seeded randomly onto Matrigel-coated nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) situated between the upper and lower well plates of each MICS chamber (six upper wells per cell line). Subsequently, the chambers were incubated with humidity for 72 hours at 37 °C. At the time of harvest, the cells that had penetrated the Matrigel-coated filters were collected, stained, and counted visually. Percent invasion was corrected for proliferation and calculated as: (total number of invading cells [lower well sample])/(total number of cells seeded [upper well sample]) × 100.

Tumor Cell Motility Assay

Unstimulated motility was determined in MICS chambers containing polycarbonate filters that had been soaked overnight in 0.1% bovine serum albumin by use of a modification of a procedure described by McCarthy et al. (*46*). Tumor cells (5×10^4) were seeded randomly in each upper well (six wells per cell line), allowed to incubate at 37 °C for 6 hours, and subsequently processed as described above for the invasion assay.

Analysis of Metastatic Potential

Cultures that were 70%-80% confluent were detached with 2 m*M* EDTA solution, and cells were resuspended in a large volume of medium. The mixture was centrifuged, and cells were resuspended in a small volume of ice-cold Hanks' balanced salt solution (HBSS). Cell counts were performed with a hemacytometer, and volumes were adjusted by addition of ice-cold HBSS. Four-week-old, athymic nude mice (Harlan Sprague-Dawley, Inc., Madison, Wis.) were inoculated subcutaneously with either 5×10^5 or 1×10^6 cells in 0.5 mL of HBSS in either the dorsolateral flank or the midscapular region. The animals were maintained and housed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were reviewed and approved by the appropriate Institutional Animal Care and Use Committees. Mice were fed Purina rodent chow and tap water (chlorine content <5 ppm) ad libitum.

Animals were killed by cervical dislocation at 27 or 62 days after inoculation, and complete gross necropsies were performed. Lungs were fixed in a neutral-buffered formalin-Bouin's fixative solution (5:1; vol/vol), and the number of surface lung metastases was counted as previously described (*47,48*).

Results

In screening the nine chosen human melanoma cell lines, we focused on the presence of specific markers (HMB-45, S-100,

vimentin, K8 and K18, and HLA-ABC class I and HLA-DR class II histocompatibility antigens) that could potentially provide some prognostic definition of "aggressive" melanomas. The information presented in Table 1 is a compilation of data derived from immunohistochemical analyses and shows that there were three consistent markers expressed in the human melanoma cell lines studied: HMB-45, vimentin, and HLA-ABC.

Specific examination of the invasive potential of the cell lines was performed in the MICS assay during a 72-hour period (Table 1). The ability of the cells to penetrate Matrigel-coated filters is presented as percent invasion ± standard error. The metastatic potential is also shown as the range and median number and reflects the ability of the cells to form spontaneous metastases in the lungs from a subcutaneous site 62 days after inoculation. Collectively, these data indicate that the C8161 cells are the most invasive and, correlatively, the most metastatic. The A375M cells are rank-ordered second in invasive and metastatic potential.

In a separate study (*42*), the abilities of A375P, A375M, and C8161 cells to form experimental metastases from intravenous injection via the tail vein in athymic nude mice were measured (data not shown). Similarly, the C8161 cells produced the highest median number of experimental lung colonies: >250 within 21 days after inoculation versus 146 for A375M and three for A375P cells 6 weeks after inoculation (*48,49*).

To continue our study of the potential role(s) for keratins in human melanoma cells, we focused our attention on (*a*) one cell line that did not express K8 and K18—A375P (low invasive and metastatic potential), (*b*) one cell line that contained subpopulations of cells expressing K8 and K18—A375M (intermediate invasive and metastatic potential), and (*c*) one cell line that strongly expressed K8 and K18—C8161 (high invasive and metastatic potential).

With indirect immunofluorescence microscopy, the localization patterns of vimentin and K8 and K18 were demonstrated, as shown in Fig. 1. Staining for vimentin was prominent in A375P, A375M, and C8161 cells (Figs. 1A, B, and C, respectively). No staining for K8 and K18 was detected in A375P cells (Fig. 1A′), and only subpopulations of cells stained in A375M cells (Fig. 1B′). However, a brilliant staining pattern for K8 and K18 was seen in C8161 cells (Fig. 1C′). In addition, sections of formalin-fixed, paraffin-embedded tissues of the metastatic lesion from which the C8161 cells were derived were positive for keratins (data not shown), indicating that the anomalous expression of keratins was not a tissue culture artifact.

Additional documentation for the presence and type of intermediate filaments in these cell lines included two-dimensional gel electrophoresis (Figs. 2A, B, and C), which demonstrated the presence of vimentin in all three cell lines. Electrophoresis showed no detectable levels of K8 and K18 in A375P and A375M cells, but it demonstrated K8 and K18 levels in C8161 cells that were similar to levels in the normal HeLa cell control (data not shown).

Western blot analysis (Fig. 3) showed low levels of K8 and K18 in A375M cells and high levels in C8161 cells. In Fig. 4, densitometric analysis of an accompanying Northern blot indicates the relative amounts of vimentin and K8 and K18 messenger (mRNA) levels: low expression in A375P (1.0), relatively higher levels in A375M (3.0 and 6.8), and the highest levels in C8161 (23 and 18).

An important enzyme associated with the metastatic behavior of several cell types is type IV collagenase (*4-10*), which has also been implicated in the directed migration of human tumor cells (*50*). We therefore measured mRNA expression levels of this 72-kd enzyme in the three selected cell lines (Fig. 5). Densitometric analysis of the Northern blots showed an increase in the relative amount of mRNA levels for A375M cells and C8161 cells compared with those for A375P (relative levels adjusted to 1.0). The corresponding levels of gelatinolytic activity of type IV collagenase, as measured by zymography, have been recently published by our laboratory (*42*). This activity correlates with the steady-state mRNA levels.

In an attempt to gather more direct evidence that keratin expression in melanoma tumor cells may contribute to an invasive-metastatic phenotype, we asked the question: What effect on invasive potential would occur in C8161 cells if their normal in-

Table 1. Presence of specific protein markers in human melanoma cell lines*

Cell line	Protein marker						Invasive potential in vitro†	Metastatic potential, subcutaneous‡
	S-100	HMB-45	K8, K18	Vimentin	HLA-ABC	HLA-DR		
UACC-456	+	+	–	+	+	–	0.03 ± 0.009	0
UACC-383	±	+	–	+	+	–	0.03 ± 0.01	0
UACC-608	–	+	–	+	+	+	0.32 ± 0.003	0
UACC-827	+	+	–	+	+	+	0.34 ± 0.07	0
A375P*	+	+	–	+	+	+	1.5 ± 0.01	0
HS294	+	+	–	+	+	+	1.5 ± 0.22	0
BOWES	+	+	–	+	+	–	3.5 ± 0.2	0
A375M*	+	+	±	+	+	+	4.2 ± 0.12	3(0-22)
C8161*	–	–	+	+	+	–	12.5 ± 0.11	31(5-187)

*Phenotyping of human melanoma cells was determined by direct and indirect immunofluorescence microscopy. + = positive marker, 60%-100% of population; – = no positive staining; and ± = subpopulation staining only.

†Invasive potential was derived by measuring percent invasion through Matrigel-coated filters in the MICS assay during 72 hours. Values = % invasion ± SE for samples per cell line.

‡Metastatic potential was evaluated by the number of spontaneous lung metastases following subcutaneous injection of 1×10^6 cells into the dorsolateral flank. Values = median (range) for six mice/group. Animals were killed 62 days after inoculation.

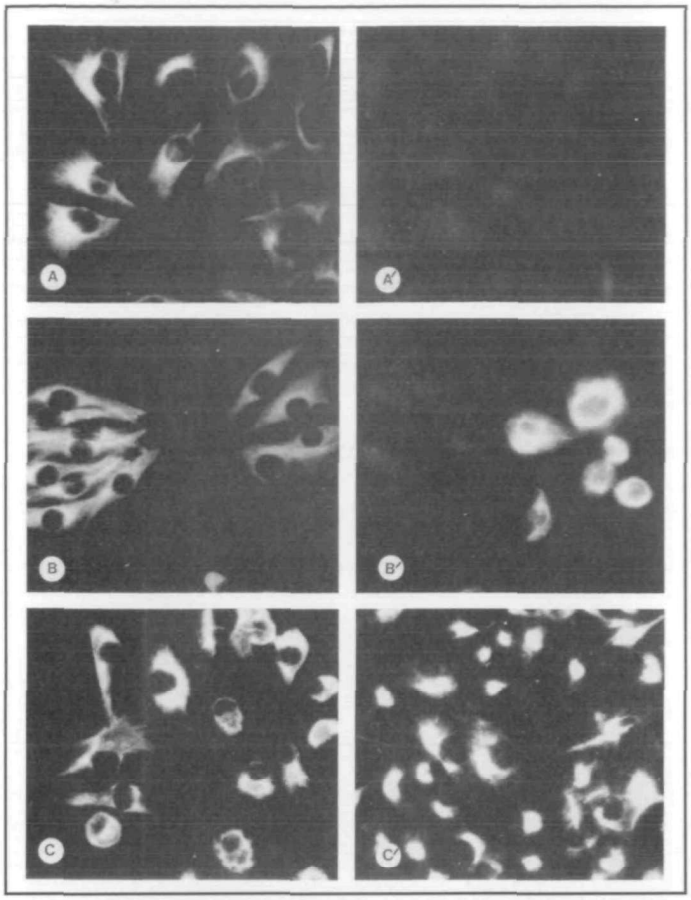

Fig. 1. Indirect immunofluorescence microscopy of human melanoma cells stained for vimentin (A,B,C) or K8 and K18 (A′,B′,C′). A375P (A), A375M (B), and C8161 (C) cells were all positive for vimentin. By comparison, keratin staining was negative in A375P (A′), positive in distinctive subpopulations in A375M (B′), and positive in all C8161 cells (C′). (original magnification ×350).

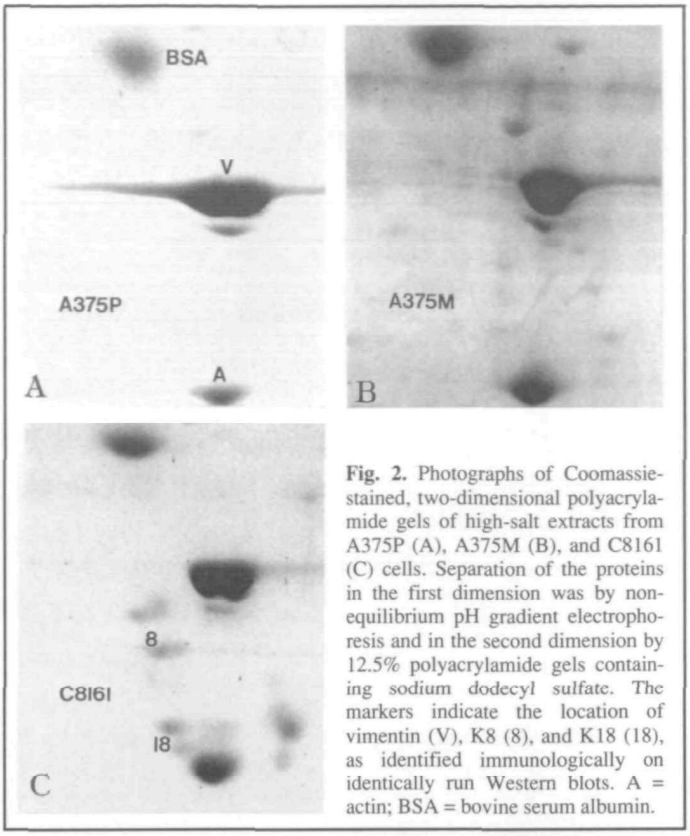

Fig. 2. Photographs of Coomassie-stained, two-dimensional polyacrylamide gels of high-salt extracts from A375P (A), A375M (B), and C8161 (C) cells. Separation of the proteins in the first dimension was by non-equilibrium pH gradient electrophoresis and in the second dimension by 12.5% polyacrylamide gels containing sodium dodecyl sulfate. The markers indicate the location of vimentin (V), K8 (8), and K18 (18), as identified immunologically on identically run Western blots. A = actin; BSA = bovine serum albumin.

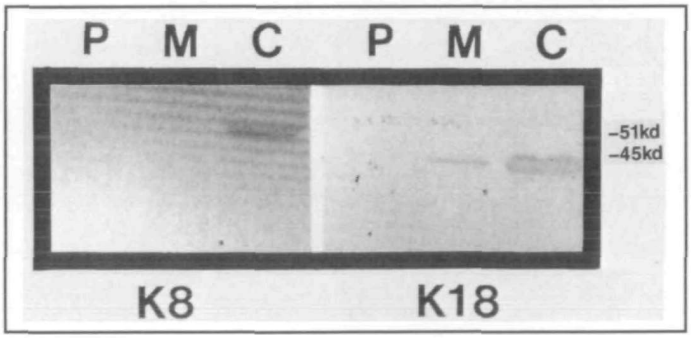

Fig. 3. Western blot analysis of K8 and K18 isolated from A375P (P), A375M (M), and C8161 (C) cells using a high-salt extraction. Ten μg of protein was loaded into each lane of the 12.5% polyacrylamide gels containing sodium dodecyl sulfate, and the separated proteins were subsequently electrophoretically transferred to an Immobilon-P transblot membrane. Detection of keratins was accomplished using either anti-K8 (left panel) or anti-K18 (right panel) primary antibodies followed by a secondary antibody conjugated with horseradish peroxidase. The molecular weight markers correspond to the putative K8 (51 kd) and K18 (45 kd).

termediate filament structure was disrupted? We addressed this question by transfecting the C8161 cells with a mutant K18 cDNA that contains only the first 5′ 1070 nucleotides. Fourteen stably transfected clones were produced. Two of these were selected for further study, and they are designated C1070-10 and C1070-14.

Indirect immunofluorescence microscopy of these clones to detect K18 demonstrated a dramatic morphological change compared with the C-NEO transfected controls, as shown in Fig. 6. The transfectant mutant cells were highly elongated and much larger than the C-NEO cells and demonstrated an intracellular staining pattern of short, discontinuous striated bundles of filaments compared with the normal morphology seen in the C-NEO controls and in the C8161 wild type (Fig. 1).

We then determined if the presence of the truncated K18 and the disruption of normal keratin filament formation in C1070-10 and C1070-14 cells affected their invasive potential (Fig. 7). Cells were tested for their ability to invade Matrigel-coated filters in the MICS assay during a 72-hour period. The C-NEO control cells demonstrated 10% invasion, similar to the 12% invasion profile of the wild-type C8161. In comparison, the C1070-10 cells showed a 3.0% invasive potential, while the C1070-14 cells achieved only a 0.4% invasive potential.

In addition, when C1070-10 and C1070-14 cells were tested for metastatic potential via subcutaneous administration, they were unable to form primary tumors or spontaneous metastases. Metastatic potential was evaluated by the size of the primary tumor as well as by the number of spontaneous lung metastases following subcutaneous injection of 5×10^5 cells into the mid-scapular region. Animals were killed 27 days after inoculation. Six of six mice receiving C-NEO cells showed primary tumors averaging 6 mm^3 and a median of three lung metastases. Six of six mice receiving C1070-10 and C1070-14 cells showed no primary tumors and no metastases.

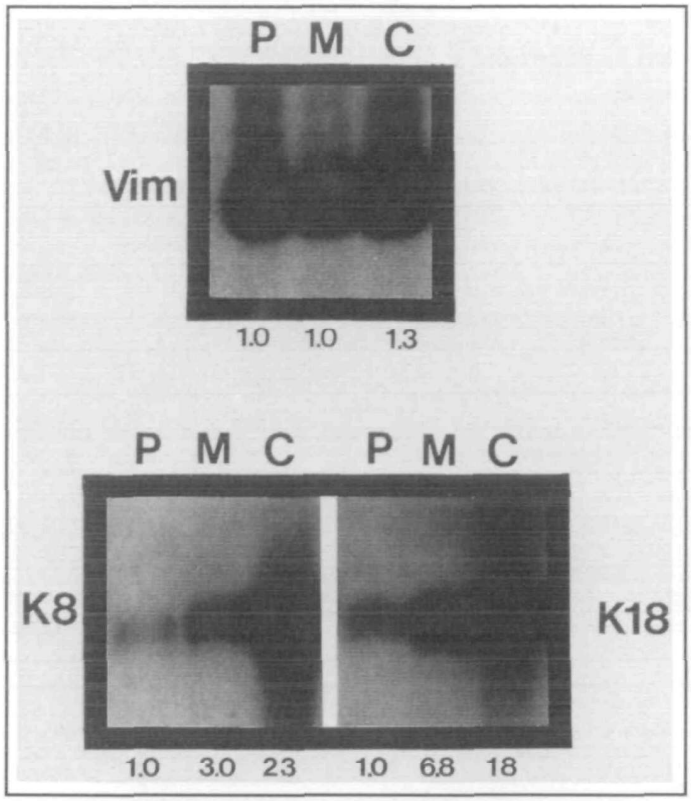

Fig. 4. Northern blot analysis of human vimentin (Vim) and human K8 and K18 in A375P (P), A375M (M), and C8161 (C) melanoma cells. Poly A selected mRNA was loaded 1 μg per lane, and relative values derived from densitometric scans are shown under each lane.

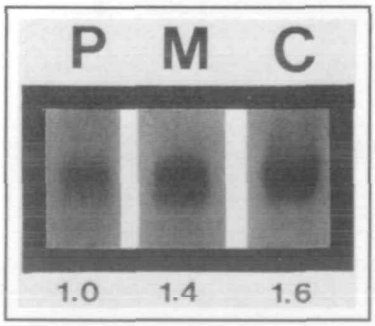

Fig. 5. Northern blot analysis of human type IV collagenase gene expression in A375P (P), A375M (M), and C8161 (C) melanoma cells. RNA was poly A selected (1 μg per lane) and fractionated on 2.2 *M* formaldehyde gels, capillary blotted to nylon membranes, and probed with ^{32}P-labeled pH3A for collagenase IV (3.2-kilobase transcript size). Relative values derived from densitometric scans are shown under each lane.

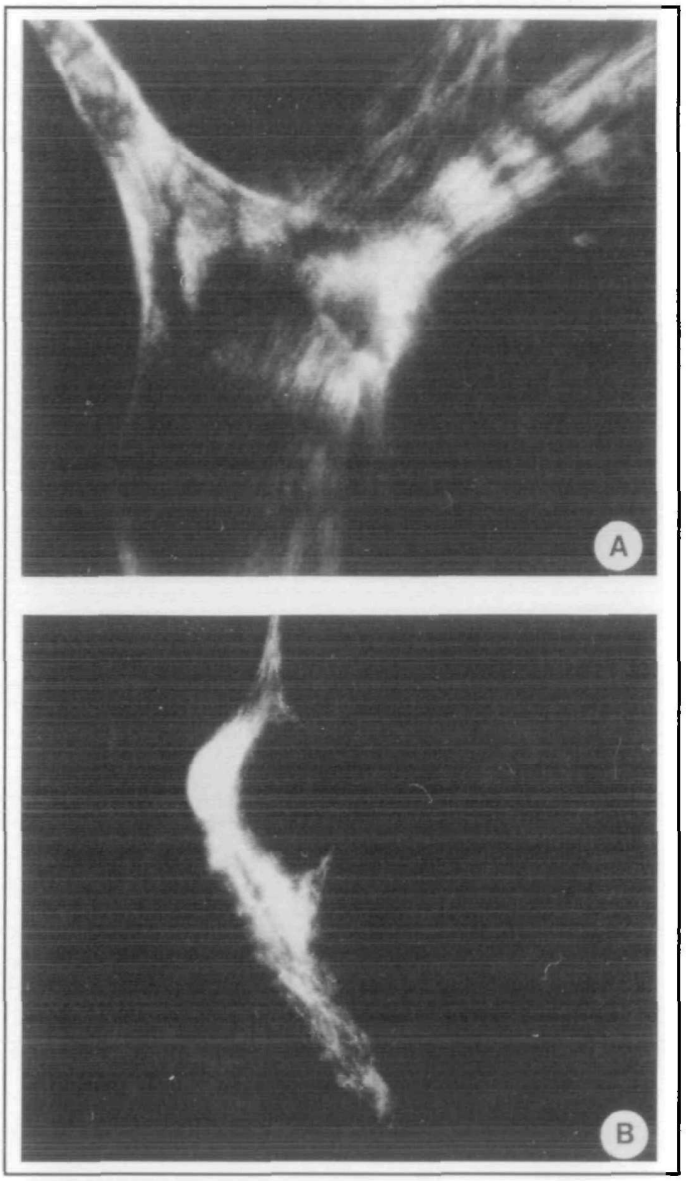

Fig. 6. Indirect immunofluorescence microscopy of K18 distribution in (A) C1070-10 cells and (B) C-NEO transfected control. (original magnification ×750).

We further investigated whether a reduction in collagenase IV gene expression could account for the dramatic decrease in invasive potential for the C1070-10 and C1070-14 cells. Zymography showed no dramatic difference between the gelatinolytic activity of the 72-kd type IV collagenase in these cells and that in the C-NEO controls (Fig. 8). Levels of mRNA shown by Northern blot analysis (data not shown) confirmed these findings.

With no significant difference shown in type IV collagenase expression, we next measured the ability of the cells to migrate through uncoated polycarbonate filters during a 6-hour period (Fig. 9). These data show a dramatic reduction in motility of both the C1070-10 and C1070-14 transfectants.

Discussion

Intermediate filaments have been implicated in vital functions related to cellular differentiation and serve as important transducers of signals to the nucleus (*18,51*). Recent advances in molecular biology have uncovered at least five distinct types of intermediate filaments expressed in various cell types, which have been ascribed a variety of important functions (*19,52*).

In our study, nine human melanoma cell lines were evaluated for the presence of the intermediate filaments vimentin and keratins K8 and K18, in addition to HMB-45, S-100, HLA-ABC, and HLA-DR. We have shown a correlation between the coexpression of vimentin with K8 and K18 and the invasive and metastatic behavior of three representative human melanoma cell lines. The poorly metastatic A375P cells showed no K8 and K18 by routine methods of two-dimensional electrophoresis with Coomassie-stained gels, Western immunoblotting, and im-

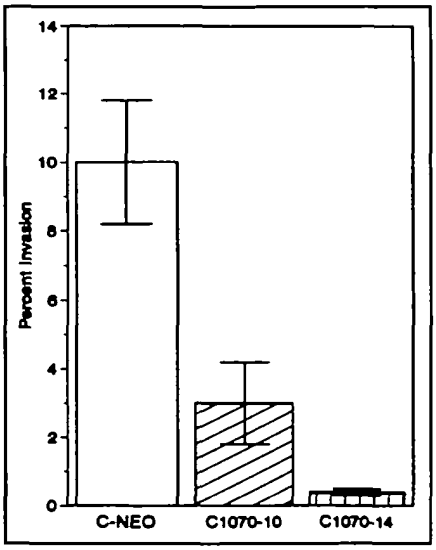

Fig. 7. Percent invasion of C-NEO control cells and C1070-10 and C1070-14 cells measured in the MICS assay during a 72-hour period using Matrigel-coated polycarbonate filters.

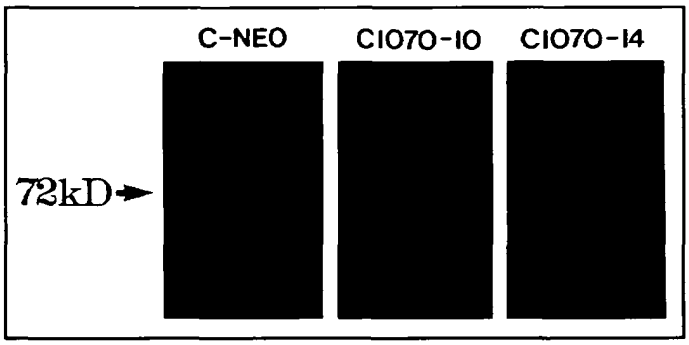

Fig. 8. Zymographic analysis of the gelatinolytic activity of the secreted 72-kd type IV collagenase from supernatants of C-NEO control cells and C1070-10 and C1070-14 cells.

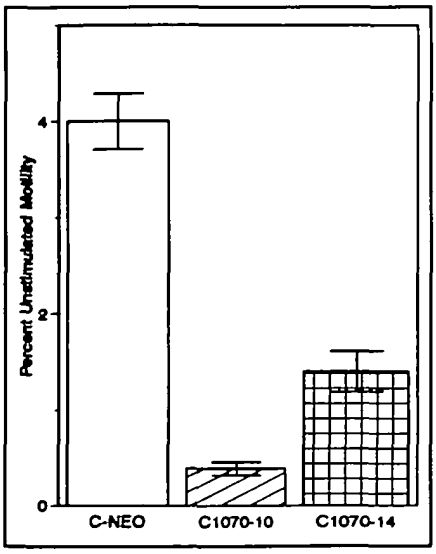

Fig. 9. Unstimulated migratory ability of C-NEO control cells and C1070-10 and C1070-14 cells measured in the MICS assay during a 6-hour period using gelatin-coated polycarbonate filters.

munofluorescence detection. However, a low level of mRNA expression of both keratins was detected by Northern blot analysis. The intermediate metastatic A375M cells showed low levels of K8 and K18 by 2-D gel electrophoresis and Western and Northern blotting, and distinctive subpopulations of cells were positive by immunofluorescence among a basically negative general population. In contrast, the highly metastatic C8161 cells showed a strong presence of K8 and K18 by Coomassie-stained gels and Western blotting and a prominent immunofluorescence staining pattern. Furthermore, Northern blot analysis showed a 10-fold increase in K8 and K18 mRNA levels in C8161 cells, compared with A375P and A375M cells.

To provide more concrete evidence for a functional role for these keratins, C8161 cells were transfected with a mutant K18 cDNA, which resulted in the disruption of normal keratin filament formation. Two of the stably transfected clones, C1070-10 and C1070-14, showed a dramatic decrease in invasive and metastatic potential, which could be attributed in part to a decrease in motility.

This anomalous expression of keratins in melanomas has recently been reported in the literature (*23-26*). In a recent study of 100 melanomas analyzed by immunohistochemistry, 21% of frozen tissue sections were shown to contain keratin, specifically in metastatic and recurrent melanomas (*53*). This same study suggested that keratin expression in melanomas may be related to tumor progression, which was demonstrated in our investigation with in vitro and in vivo models. In another report characterizing a highly heterogeneous human melanoma cell line (IIB-MEL-J), three subpopulations were isolated by Percoll gradient centrifugation, and all were shown to express keratin-positive intermediate filaments (*54*). A third study found that one of nine melanoma cell lines specifically expressed keratins 7,8,13,17, and 18 (*55*).

In these reports demonstrating the coexistence of keratins and vimentins, three important variables are apparent which potentially confound the pathologist's diagnosis of melanoma versus numerous types of carcinomas: (*a*) the fixation of the tissue sample, (*b*) the mode of detection, and (*c*) the interpretation of the data generated. It appears that the best diagnosis could be made by using a panel of monoclonal antibodies to HMB-45, S-100, keratins, and vimentin, in combination with Western immunoblotting and Northern blot analysis or in situ hybridization for further investigation. At the molecular level, important information relating to gene regulation could be correlated with structure and function.

In our study, all cell lines were positive for HMB-45, which identifies a premelanosomal glycoprotein found in activated and neoplastic melanocytes and differentiates keratin-positive melanomas from carcinomas (*11*). In addition, all cell lines were positive for vimentin, which is a classical marker for melanoma (*12*). The expression of keratins in A375M cell subpopulations may reflect tumor cell heterogeneity (*56-58*) and the development of clones with distinct biological differences. A possible explanation for this phenomenon has been offered by Knapp and Franke (*59*). These investigators suggest that an intrinsic instability of the inactive state of the K8 and K18 genes is responsible for the occurrence of these protein products in certain nonepithelial tissues and tumors. Specifically, Knapp and Franke found that several different transformed nonepithelial cells constitutively express K8 and K18 and that these genes can be transcribed without an accumulation of detectable amounts of mRNA and without the appearance of keratin-containing filaments (*59*). These data reinforce the need for using more than

one technique in evaluating the presence of specific intermediate filaments, which was demonstrated in our study.

In attempting to draw a correlation between keratin expression and properties associated with invasive and metastatic behavior as well as directed migration (*4-10,50*), we measured the expression of type IV collagenase. By Northern blot analysis, mRNA levels of the enzyme are lowest in A375P cells and highest in C8161 cells. To show a more direct relationship between keratin expression and invasive and metastatic behavior, we used C1070-10 and C1070-14 cells containing a stably transfected mutant K18 (*60*) in this study.

There are at least 19 separate keratins ranging in size from 40 to 68 kd. Keratins are commonly expressed as particular pairs, which form heteropolymers in cells. For example, K18 is normally coexpressed with K8 to form a structural filament bundle. Transfection of a mutant K18 protein into the highly invasive C8161 cells would likely disrupt the normal interactions between K8 and K18. Unfortunately, there is no specific antigenic epitope on the K18 mutant protein that does not also appear on the endogenous K18. Therefore, it is not possible to differentiate the localization of K18 mutant protein from normal K18 protein in the cells. Since the carboxy-terminal deletion on K18 includes the most conserved part of the coil 2 structure that is shared with all intermediate filament proteins and the tail region, it is possible that this deletion could cause the failure of certain regions on the K8 protein to complex with their normal pairing region, resulting in discontinuous filament formation. This conformational change in filament bundling could account for alterations in signal transduction, thus affecting invasive and metastatic ability.

In a previous study, Albers and Fuchs (*61*) showed that the transient expression of a truncated mutant keratin cDNA in simple epithelial cells and squamous carcinoma also resulted in disruption in the keratin filament network. Furthermore, additional evidence implicating the involvement of K8 and K18 in cellular migration and invasion has recently been reported by our laboratory using the mouse L cell model, in which fibroblastic cells transfected with K8 and K18 were at least 10-fold more invasive and migratory than the parental cells (*62*).

When type IV collagenase activity was investigated to provide evidence of a potential mechanism that might be responsible for the reduction in invasive activity, we discovered that the mRNA level of expression and the gelatinolytic activity of this enzyme were no different in the C-NEO control cells and the C1070-10 and C1070-14 clones. When unstimulated migratory ability was analyzed, there was a commensurate drop in migration of the C1070-10 and C1070-14 cells versus the C-NEO control cells, which may, in part, explain the inability of the C1070-10 and C1070-14 cells to metastasize. However, there was no detection of even a primary tumor, which should be further investigated from the viewpoint of host immunological factors.

In trying to postulate potential role(s) for the expression of keratins in melanomas, it is tempting to correlate the following facts: (*a*) Keratins are the first intermediate filaments to be expressed during embryogenesis. Therefore, epithelial as well as nonepithelial cellular derivatives originate from these precursors (*63*). (*b*) K8 and K18 are complementary and are observed in "simple epithelium" of many early embryonic and certain adult tissues (*64*). (*c*) Keratins have been detected in certain sarcomas (*65*), astrocytomas (*66*), smooth muscle tumors (*67*), and various other nonepithelial tumors [*see* review in (*68*)].

Collectively, these data reinforce the suggestion by Trejdosiewicz et al. (*55*) that expression of keratins in neoplastic cells of neuroectodermal origin represents a reversion to a more primitive, embryonic phenotype. This "dedifferentiated" state is compatible with a more uninhibited phenotype capable of adapting to mesenchymal as well as epithelial matrices and thus able to interpret additional positional clues which may contribute to a more aggressive behavior (*69*). Taking into consideration the fact that intermediate filaments can act as signal transducers, relaying information from the extracellular matrix to the nucleus (*51*), and that matrix can regulate gene expression (*70,71*), it is tempting to speculate that the ability to coexpress vimentin and keratins offers a selective advantage to tumor cells. Indeed, a general theme is beginning to emerge that suggests the phenomenon of "switching" phenotypic expression and coexpression of two phenotypic markers in cells confers a selective advantage toward transformation. Understanding the genetic regulation of this anomalous expression holds the key to more efficient diagnoses and more effective chemotherapeutic strategies.

References

(*1*) Rigel D, Kopf AW, Friedman RJ: The rate of malignant melanoma in the United States: Are we making an impact? J Am Acad Dermatol 17:1050–1053, 1987

(*2*) Akslen LA, Hove LM, Hartveit F: Metastatic distribution in malignant melanoma: A 30-year autopsy study. Invasion Metastasis 7:253–263, 1987

(*3*) Paul E, Pausch A, Bodeker RH: Speed of growth of melanoma: Statistical analysis of the average ages of patient groups. Pigment Cell Res 2:475–477, 1989

(*4*) Liotta LA, Guirguis R, Stracke M: Biology of melanoma invasion and metastasis. Pigment Cell Res 1:5–15, 1987

(*5*) Liotta LA, Rao CN, Wewer UM: Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 55:1037–1057, 1986

(*6*) Stetler-Stevenson WG, Krutzsch HC, Wacher MP: The activation of human type-IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. J Biol Chem 264:1353–1356, 1989

(*7*) Reich R, Thompson EW, Iwamoto Y, et al: Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res 48:3307–3312, 1988

(*8*) Wang M, Stearns ME: Blocking of collagenase secretion by estramustine during in vitro tumor cell invasion. Cancer Res 48:6262–6271, 1988

(*9*) Mignatti P, Robbins E, Rifkin DB: Tumor invasion through the human amniotic membrane: Requirement for a proteinase cascade. Cell 47:487–498, 1986

(*10*) Ura H, Bonfil RD, Reich R, et al: Expression of type-IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells. Cancer Res 49:4615–4621, 1989

(*11*) Ordonez NG, Ji XL, Hickey RC: Comparison of HMB-45 monoclonal antibody and S-100 protein in the immunohistochemical diagnosis of melanoma. Am J Clin Pathol 90:385–390, 1988

(*12*) Caselitz J, Breitbart E, Weber K, et al: Malignant melanomas contain only the vimentin-type of intermediate filaments. Virchows Arch 400:43–50, 1983

(*13*) Huszar M, Halkin H, Herczeg E, et al: Use of antibodies to intermediate filaments in the diagnosis of metastatic amelanotic malignant melanoma. Hum Pathol 14:1006–1008, 1983

(*14*) Miettinen M, Lehto V-P, Virtanen I: Presence of fibroblast-type of intermediate filaments (vimentin) and absence of neurofilaments in pigmented nevi and melanomas. J Cutan Pathol 10:188–192, 1983

(15) GOWN AM, VOGEL AM, HOAK D, ET AL: Monoclonal antibodies specific for melanocytic tumors distinguish subpopulations of melanocytes. Am J Pathol 123:195–203, 1986
(16) HOUGHTON AN, ALBINO AP, CORDON-CARDO C, ET AL: Cell surface antigens of human melanocytes and melanoma. J Exp Med 167:197–212, 1988
(17) MACKIE RM, CAMPBELL I, TURBITT ML: Use of NK1 C3 monoclonal antibody in the assessment of benign and malignant melanocytic lesions. J Clin Pathol 37:367–372, 1984
(18) STEINERT PM, LIEM RK: Intermediate filament dynamics. Cell 60:521–523, 1990
(19) NAGLE RB: Intermediate filaments: A review of the basic biology. Am J Surg Pathol 12:4–16, 1988
(20) FUCHS E, HANUKOGLU I: Unraveling the structure of the intermediate filaments. Cell 34:332–334, 1983
(21) OSBORN M, WEBER K: Intermediate filaments: Cell-type-specific markers in differentiation and pathology. Cell 31:303–306, 1982
(22) FRANKE WW, SCHMID E, MOLL R: The intermediate filament cytoskeleton in tissues and in cultured cells: Differentiation specificity of expression of cell architectural elements. *In* Human Carcinogenesis (Harris CC, Autrup HN, eds). New York: Academic Press, 1983, pp 3–34
(23) MIETTINEN M, FRANSSILA K: Immunohistochemical spectrum of malignant melanoma: The common presence of keratins. Lab Invest 61:623–628, 1989
(24) AZUMI N, BATTIFORA H: The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. A comprehensive immunohistochemical study on formalin and alcohol fixed tumors. Am J Clin Pathol 88:286–296, 1987
(25) CHAN R, ROSSITTO PV, EDWARDS BF, ET AL: Presence of proteolytically processed keratin in the culture medium of MCF-7 cells. Cancer Res 46:6353–6359, 1986
(26) GATTER KC, RALFKIAER E, SKINNER J, ET AL: An immunocytochemical study of malignant melanoma and its differential diagnosis from other malignant tumors. J Clin Pathol 38:1353–1357, 1985
(27) KOZLOWSKI JM, HART IR, FIDLER IJ, ET AL: A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. J Natl Cancer Inst 72:913–917, 1984
(28) BREGMAN MD, FUNK C, FUKUSHIMA M: In vitro modulation of human and murine melanoma growth by prostanoid analogs. Prostaglandins 27:17–26, 1983
(29) CREASEY AA, SMITH HS, HACKETT AJ, ET AL: Biological properties of human melanoma cells in culture. In Vitro 15:342–350, 1979
(30) RIFKIN DB, LOEB JN, MOORE G, ET AL: Properties of plasminogen activators formed by neoplastic human cell cultures. J Exp Med 139:1317–1328, 1974
(31) KULESH DA, CECENA G, DARMON YM, ET AL: Posttranslational regulation of keratins: Degradation of mouse and human keratins 18 and 8. Mol Cell Biol 9:1553–1565, 1989
(32) GUNNING P, LEAVITT J, MUSCAT G, ET AL: A human β-actin expression vector system directs high-level accumulation of antisense transcripts. Proc Natl Acad Sci USA 84:4831–4835, 1987
(33) GRAHAM FL, VAN DER EB J: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467, 1973
(34) KINGSTON RE, KAUFMAN RJ, SHARP PA: Regulation of transcription of the adenovirus EII promoter by EI gene products: Absence of sequence specificity. Mol Cell Biol 4:1970–1977, 1984
(35) TRENT JM, ROSENFELD SB, MEYSKENS FL: Chromosome 6q involvement in human malignant melanoma. Cancer Genet Cytogenet 9:177–180, 1983
(36) O'FARRELL PZ, GOODMAN HM, O'FARRELL PH: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133–1141, 1977
(37) LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
(38) KINDBLOM LG, LODDING P, ROSENGREN L, ET AL: S-100 protein in melanocytic tumors. An immunohistochemical investigation of benign and malignant melanocytic tumors and metastases of malignant melanoma and a characterization of the antigen in comparison to human brain. Acta Pathol Microbiol Immunol Scand 92:219–230, 1984
(39) GOWN AM, VOGEL AM: Monoclonal antibodies to human intermediate filament proteins. II. Distribution of filament proteins in normal human tissues. Am J Pathol 114:309–321, 1984
(40) CHIRGWIN JM, PRZYBYLA AE, MACDONALD RJ, ET AL: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299, 1979
(41) LEHRACH H, DIAMOND D, WOZNEY JM, ET AL: RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16:4743–4751, 1977
(42) LEVY AT, CIOCE V, SOBEL ME, ET AL: Increased expression of the Mr 72 000 type IV collagenase in human colon adenocarcinomas. Cancer Res 51:439–444, 1991
(43) FERRARI S, BATTINI R, KACZMARE KL, ET AL: Coding sequence and growth regulation of the human vimentin gene. Mol Cell Biol 11:3614–3620, 1986
(44) HENDRIX MJ, SEFTOR EA, SEFTOR REB, ET AL: A simple quantitative assay for studying the invasive potential of high and low human metastatic variants. Cancer Lett 38:137–147, 1987
(45) KLEINMAN HK, MCGARVEY ML, LIOTTA LA, ET AL: Isolation and characterization of type IV procollagen, laminin and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21:6188–6193, 1982
(46) MCCARTHY JB, PALM SL, FURCHT LT: Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. J Cell Biol 97:772–777, 1983
(47) WELCH DR, NERI A, NICOLSON GL: Comparison of "spontaneous" and "experimental" metastases using rat 13762 mammary adenocarcinoma metastatic cell clones. Invasion Metastasis 3:65–80, 1983
(48) WELCH DR, BISI JE, MILLER BE, ET AL: Characterization of a highly invasive and spontaneously metastatic human malignant melanoma cell line. Int J Cancer 47:227–237, 1991
(49) HENDRIX MJC, SEFTOR EA, SEFTOR REB, ET AL: Comparison of tumor cell invasion assays: Human amnion versus reconstituted basement membrane barriers. Invasion Metas 9:278–297, 1989
(50) TERRANOVA VP, MASLOW D, MARKUS G: Directed migration of murine and human tumor cells to collagenases and other proteases. Cancer Res 49:4835–4841, 1989
(51) GOLDMAN RD, GOLDMAN AE, GREEN KJ: Intermediate filament networks: Organization and possible functions of a diverse group of cytoskeletal elements. J Cell Sci 5:69–97, 1986
(52) STEINERT PM, ROOP DR: Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 57:593–625, 1988
(53) ZARBO RJ, GOWN AM, NAGLE RB, ET AL: Anomalous cytokeratin expression in malignant melanoma: One- and two-dimensional Western blot analysis and immunohistochemical survey of 100 melanomas. Mod Pathol 3:494–501, 1990
(54) GUERRA L, MORDOH J, SLAVUTSKY I, ET AL: Characterization of IIB-MEL-J: A new and highly heterogeneous human melanoma cell line. Pigment Cell Res 2:504–509, 1989
(55) TREJDOSIEWICZ LK, SOUTHGATE J, KEMSHEAD JT, ET AL: Phenotypic analysis of cultured melanoma cells: Expression of cytokeratin-type intermediate filaments by the M5 human melanoma cell line. Ex Cell Res 164:388–398, 1986
(56) FIDLER IJ, HART IR: Biological diversity in metastatic neoplasms: Origins and implications. Science 217:998–1003, 1982
(57) NICOLSON GL: Cancer metastasis: Tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim Biophys Acta 948:175–224, 1988
(58) HOUGHTON AN, REAL FX, DAVIS LJ, ET AL: Phenotypic heterogeneity of melanoma. Relation to the differentiation program of melanoma cells. J Exp Med 165:812–829, 1987
(59) KNAPP AC, FRANKE WW: Spontaneous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occurring at different levels of regulation. Cell 59:67–79, 1989
(60) CHU YW, DUFFY JJ, SEFTOR REB, ET AL: Disruption of normal cytokeratin formation by the transfection of a deleted CK18 cDNA into a highly metastatic melanoma cell line decreases the invasive potential. J Clin Biotech 3:27–33, 1991
(61) ALBERS K, FUCHS E: The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. J Cell Biol 105:791–806, 1987
(62) CHU YW, OSHIMA RG, HENDRIX MJ: Expression of keratins 8 and 18 in mouse L cells results in an increase in migratory and invasive ability. J Cell Biol 111:42A, 1990
(63) FRANKE WW, GRUND C, KUHN C, ET AL: Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. Differentiation 23:43–49, 1982
(64) JACKSON BW, GRUND C, SCHMID E, ET AL: Formation of cytoskeletal elements during mouse embryogenesis. Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. Differentiation 17:161–179, 1980
(65) MIETTINEN M, RAPOLA J: Immunohistochemical spectrum of rhabdomyosarcoma and rhabdomyosarcoma-like tumors. Expression of cytokeratin and 68-kd neurofilament protein. Am J Surg Pathol 13:120–132, 1989
(66) COSGROVE M, FITZGIBBONS PL, SHERROD A, ET AL: Intermediate filament expression in astrocytic neoplasms. Am J Surg Pathol 13:141–145, 1989

(67) Brown DC, Theaker JM, Banks PM, et al.: Cytokeratin expression in smooth muscle and smooth muscle tumors. Histopathology 11:477–486, 1987

(68) Franke WW, Jahn L, Knapp AC: Cytokeratins and desmosomal proteins in certain epithelioid and non-epithelial cells. *In* Cytoskeletal Proteins in Tumor Diagnosis (Osborn M, Weber K, eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989, pp 151–172

(69) Hendrix MJC, Saba PZ, McDaniel KM, et al.: Culturing human melanoma cells of varying metastatic potential on different substrata can influence invasive potential. J Cell Biol 107:797A, 1988

(70) McDonald JA: Matrix regulation of cell shape and gene expression. Curr Opin Cell Biol 1:995–999, 1989

(71) Hay ED: Theory for epithelial-mesenchymal transformation based on the "fixed cortex" cell motility model. Cell Motil Cytoskeleton 14:455–457, 1989